Cumhuriyet Sci. J., 43(4) (2022) 645-651
DOI: https://doi.org/10.17776/csj.1114125

Cumhuriyet Science Journal

| csj.cumhuriyet.edu.tr | Founded: 2002 ISSN: 2587-2680 e-ISSN: 2587-246X Publisher: Sivas Cumhuriyet University

Simultaneous Manipulation and Imaging of Chemogenetically Induced Hydrogen Peroxide in Hardly Transfectable Endothelial Cells

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Research Article

History
Received: 09/05/2022
Accepted: 28/09/2022

ABSTRACT

Hydrogen peroxide (H_2O_2) is a critical signaling molecule in vascular cells, which controls signaling events, yet it can cause pathological oxidative stress in excess. The lack of suitable tools undermined experimental approaches to study the role of oxidative eu- and distress in cellular ultra-locales. This study exploits a yeast-derived D-amino acid oxidase (mDAAO) as a chemogenetic tool to induce, visualize and test the cytotoxicity of H_2O_2 in hardly transfectable endothelial cells. Due to the poor transfectability of endothelial cells, lentiviral vectors have been used to generate cell lines stably expressing mDAAOs. mDAAOs are substrate-based chemogenetic enzymes that convert D-amino acids to their corresponding alpha-keto acids and generate H_2O_2 as a byproduct, which can be visualized with a novel ultrasensitive, and ratiometric H_2O_2 biosensor termed HyPer7. This study tested the suitability of two different D-amino acids, including D-alanine and D-methionine, to induce oxidative stress in endothelial cells. Live-cell imaging experiments unveiled that 10 mM D-methionine generated significantly higher and faster H_2O_2 signals than D-alanine. However, both D-amino acids induced comparable levels of cell death documented by a colorimetric cell metabolic activity assay (MTT). This study provides a guide for manipulating and monitoring the cytotoxic effect of H_2O_2 in endothelial cells.

Keywords: Chemogenetic tools, Endothelial cells, Hydrogen Peroxide, Genetically Encoded Biosensors, Cytotoxicity

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Introduction

Oxidative stress is characterized by the imbalance between sufficient generation and abundant accumulation of reactive oxygen species (ROS) in cells and tissues [1], which is a hallmark of many cardio- and neurovascular diseases like heart failure, atherosclerosis, ischemia, stroke, diabetes Mellitus, and Alzheimer's disease or dementia [2], all leading causes of death globally [3]. It has been established that increased levels of oxidative stress are implicated in the progression of cardio- and neurovascular diseases [4,5]. Thus, ROS have long been recognized as destructive molecules [6]. A more recent concept defines oxidative eustress as physiological oxidative stress required and essential for redox-dependent signaling pathways [7]. The relatively stable ROS hydrogen peroxide (H_2O_2) fine-tunes kinase-driven molecules such to control cell division, differentiation, and migration by reversible cysteine oxidation of these key signaling proteins [8]. However, excessive oxidative stress causes disturbed redox signaling and oxidative damage to macromolecules, termed oxidative distress, and the underlying mechanisms causative for the transition from healthy conditions to pathogenesis are not fully understood yet [9].

Potential sources of ROS for cardio- and neurovascular diseases can be found in all layers throughout the vasculature [10]. Of particular interest are endothelial cells, which form the inner layer of blood- and lymphatic vessels. Endothelial cells are rich in mitochondria and NADPH oxidase (NOX), two critical sources for ROS generation [11]. ROS are usually produced by xanthine oxidase, nicotinamide adenine dinucleotide phosphate oxidase, lipoxygenases, or uncoupling endothelial nitric oxide synthase (eNOS) in vascular cells [12]. Any imbalance in the equilibrium between the production of reactive oxidants and antioxidant capacity triggers oxidative distress, which in turn leads to the progression of pathological conditions [13]. Thus, informative tools and technologies permitting real-time monitoring of ROS-dependent pathways are of the highest interest.

Genetically encodable biosensors are fluorescent protein-based nano-probes allowing the selective and ultrasensitive detection of their analytes in cellular ultra-locales [14-17]. Recent advances in the real-time detection and quantification of ROS levels with the aid of genetically encoded biosensors unveiled that spatial and temporal sources of ROS and reactive nitrogen species (RNS) have diverse functions within the cell [18-21]. Ever since the development of the first genetically encoded ROS, RNS, and reactive sulfur species (RSS) biosensors, much has been learned about the ultra-local role of these reactive molecules. However, the lack of suitable tools to manipulate the redox tone in subcellular locales undermined recapitulating in vivo conditions in cells and tissues. Conventional approaches, which administer physiologically irrelevant concentrations of H_2O_2 in micro-

and millimolar range directly to cells, failed to recapitulate H_2O_2-derived signaling pathways [20,22,23].

In recent years, the toolkit of redox biologists has been expanded by a yeast-derived enzyme D-amino acid oxidase (DAAO), which turned useful as a chemogenetic tool permitting the manipulation of redox levels on the level of tissues, cells, and even subcellular locales with high precision [22-24]. Chemogenetics refers to an experimental system that becomes activated in the presence of its substrate and remains silent in the absence of its biochemical stimulus [25]. In a recent study, our lab established and fine-tuned a mutated version of DAAOs, termed mDAAO, with improved enzymatic activity that is capable of efficiently converting various D-amino acids into their corresponding alpha-keto acids, producing the mild oxidant H_2O_2, ammonia (NH_4^+), and pyruvate in the presence of molecular oxygen and the cofactor flavin adenine dinucleotide (FAD) (Figure 1A). Because both NH_4^+ and pyruvate are usually abundant in cells, the amount of these byproducts produced is negligible [25]. One mole of D-amino acid produces equimolar concentrations of H_2O_2 [26]. The type of D-amino acid and the amino acid uptake efficiency dictates the intracellular generation of mDAAO's byproducts (Figure 1A), which typically yields H_2O_2 in the lower micromolar range [24]. Pairing chemogenetic enzymes with genetically encoded biosensors allow multiparametric imaging and manipulation of cells and tissues with ultrahigh precision (Figure 1 B). mDAAOs are genetically encodable; thus, these enzymes can be tagged with fluorescent proteins and differentially targeted to defined subcellular locales such as the cytosol, nucleus, or mitochondria (Figure 1C) using leading peptides allowing manipulation of the redox state with a high spatial resolution.

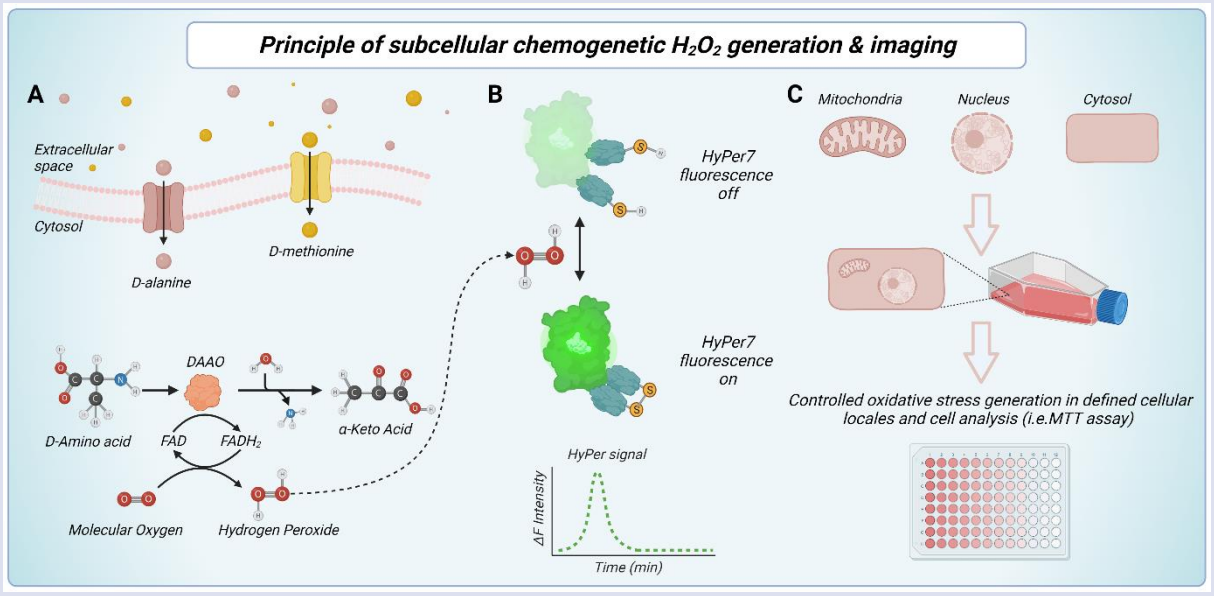

Figure 1: Schematic overview of the chemogenetic approach: (A) D-amino acid uptake depends on the expression rate of amino acid transporters and the availability of L-amino acids in the extracellular medium. The lower part of panel A shows the mDAAO pathway. (B) Schematic representation of chemogenetically induced H2O2 detection with the biosensor HyPer7. (C) Potential targeting sites of mDAAOs in subcellular locales and oxidative stress generation to assay cell viability.

This study employs these powerful approaches in hardly transfectable cells and guides how to manipulate and visualize H_2O_2 levels in subcellular locales of the immortalized human umbilical vein endothelial cell (HUVEC) line EA.hy926 [27]. Particularly, HUVECs, and its immortalized cell lines are accepted as hard-to-transfect primary cells, even with the most used transfection techniques because of their slow division [28]. Besides simultaneous generation and imaging of H_2O_2 signals in cells, this study also unveils the cytotoxic role of cytosolic H_2O_2 generation in endothelial cells using different D-amino acids.

Materials and Methods

Molecular Cloning

The differentially targeted chimera mCherry-mDAAO were subcloned into a 3rd-generation lentivirus shuttle vector pLenti-MP2 (Addgene #36097) via PCR techniques using the primers: forward 5'-ATACTCGAGATGGTGAGCAAGGGCGAG-3' including XhoI restriction site and reverse 5'-ATATCTAGATTACAGGGTCAGCCGCTC-3' including a stop codon and XbaI restriction site. HyPer7 [15] biosensor was a kind gift from the Vsevolod Belousov and is available at Addgene (#136467).

Cell Culture

HEK293T and EA.hy926 cells (ATCC, CRL-2922, Manassas, VA, USA) cells were grown in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Pan-Biotech, Aidenbach, Germany), 100 µg/mL Penicillin (Pan-Biotech, Aidenbach, Germany), and 100 U/mL Streptomycin (Pan-Biotech, Aidenbach, Germany). EA.hy926 cells were additionally supplemented with 100 µg/mL Normocin (InvivoGen, San Diego, CA, USA), and 2% HAT ((Sodium Hypoxanthine (5 mM), Aminopterin (20 µM), and Thymidine (0.8 mM)) (ATCC, VA, USA). Cells were maintained in a humidified CO_2 chamber (5% CO_2, 37 ◦C).

Lenti Virus Generation

At a confluency of 80–90%, HEK293T cells were co-transfected with 3 µg psPAX2 (Addgene #12260), 3 µg pMD2.G (Addgene #12259), and 6 µg of mCherry-mDAAO-NES lentivirus shuttle vector using PolyJet transfection reagent (SignaGen Laboratories, Rockville, MD, USA). Following 24 hours posttransfection, the culture medium was replaced by fresh DMEM. Cells were further incubated for 24 h and 48 h before collecting the cell culture medium containing virus particles. The virus-containing culture medium was filtered using a 0.45 µm filter (TPP, Switzerland) to concentrate the lentivirus particles. A 100 kDa Amicon® Ultra15 Centrifugal Filter Unit was used for subsequent ultra-filtration at 3000× g, for 30 min, at 4 ◦C). Concentrated virus particles were aliquoted and used immediately or stored at −80 ◦C.

Stable Endothelial Cell Line Generation and Transient Transfection

Stable cell line generation was conducted following the guidelines as described elsewhere [27]. Briefly, EA.hy926 cells were seeded on a 30 mm well plate 24 hours before virus administration. At 50% - 60% confluency, the complete medium was replaced by an antibiotic-free transduction medium containing 10% FBS, 10 µg/mL Polybrene infection reagent (Sigma-Aldrich, St. Louis, MO, USA), and the lentivirus particles encoding for cytosolic targeted mCherry-mDAAO. Cells were incubated in the virus-containing medium for 48–72 h. Positive transduction was determined using conventional epifluorescence microscopy. If cell transduction was positive, cells were cultured for one week in fresh complete DMEM on a 10 cm culture dish. Fluorescence activated cell sorting (FACS) was used to select mCherry-mDAAO positive cells by detecting red fluorescence emission using an excitation wavelength of 555 nm laser (Filter type: BP 555/30 nm) in a BD-Influx Cell Sorter. Cells were then grown under standard culture conditions. Before imaging experiments, cells were seeded on a 30 mm glass coverslip (Glaswarenfabrik Karl Knecht Sondheim, Sondheim vor der Rhön, Germany) 24 hours before the experiment. For transient transfection of EA.hy926 cells, 1 µg of purified plasmid Hyper7 was used with 2.5 µL PolyJet transfection reagent according to manufacturer's instructions.

Imaging Buffers and Solutions

If not otherwise stated, all chemicals were purchased from NeoFroxx. Live-cell imaging experiments using gravity-based perfusion systems were conducted as described elsewhere [28,29]. Briefly, a cell storage buffer was used to adapt cells to a HEPES-buffered solution at room temperature containing 2 mM $CaCl_2$, 5 mM KCl, 138 mM NaCl, 1 mM MgCl, 1 mM HEPES (Pan-Biotech, Aidenbach, Germany), 0.44 mM KH_2PO_4, 2.6 mM $NaHCO_3$, 0.34 mM NaH_2PO_4, 10 mM D-Glucose, 0.1% MEM Vitamins (Pan-Biotech, Aidenbach, Germany), 0.2% essential amino acids (Pan-Biotech, Aidenbach, Germany), 100 µg/mL Penicillin (Pan-Biotech, Aidenbach, Germany), and 100 U/mL Streptomycin (Pan-Biotech, Aidenbach, Germany). Buffer pH was adjusted to 7.43 using 1 M NaOH and sterilized using a 0.45 µm medium filter (Isolab, Germany). For real-time imaging, a HEPES-buffered physiological salt solution was freshly prepared before each imaging experiment consisting of 2 mM $CaCl_2$, 5 mM KCl, 138 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM D-Glucose, and pH was adjusted to 7.43 using 1 M NaOH. All imaging buffers have been prepared and used immediately before imaging experiments.

Fluorescence Microscopy

Imaging was performed on an inverted widefield epifluorescence microscope Zeiss Axio Observer.Z1/7 (Carl Zeiss AG, Oberkochen, Germany). All experiments were performed using a PlanApochromat 20×/0.8 dry objective. The microscope was equipped with an LED. Light source Colibri 7 (423/44 nm, 469/38 nm, 555/30) for HyPer and mCherry imaging and a monochrome CCD. Camera Axiocam 503 for image acquisition. HyPer7 signals were collected by alternately exciting cells using a motorized dual-filter wheel decorated with beam splitters (FT455 (for HyPer low, F420) and FT495 (for HyPer high, F490)). Emissions were alternately collected using a bandpass filter (BP 525/50). mCherry-mDAAO signals were collected using the filter combinations FT570 (BS) and emission filter 605/70. Zen Blue 3.1 Pro software (Carl Zeiss AG, Oberkochen, Germany) was used for control and data acquisition. A custom-made gravity-based perfusion system was used to administer and withdraw D-amino acids or H_2O_2.

MTT (3-(4,5-dimethylthiazol-2-YL)-2,5-diphenyltetrazolium bromide) Assay

Cells were incubated for 24 hours with different concentrations of D-methionine or D-alanine, as described in the results section. Following cell treatment, 100 µl fresh culture medium and 10 µL of a 12 mM MTT stock solution was added to each well of a 96-well plate. Cells were further incubated at 37 °C for 4 hours. Culture medium except 25 µL was removed and 50 µL of DMSO was added to each well and mixed thoroughly with the pipette to dissolve the dye. Cells were further incubated at 37 °C for 10 minutes. The absorbance was determined spectrophotometrically at 540 nm using a reference

wavelength of 630 nm on a Tecan microplate reader Infinite 200 pro (DKSH, USA).

Statistics

GraphPad Prism software version 5.04 (GraphPad Software, San Diego, CA, USA) was used for data analysis. All experiments were performed at least in triplicates. Student's t-test was applied to test significant differences between group means. Statistical significance was considered meaningful if the p-value summary was $p<0.0001$.

Results and Discussion

Endothelial cells are hardly transfectable cells with conventional transfection reagents [30]. Therefore, this study generated EA.hy926 cell lines stably expressing yeast-derived (from *R. gracilis*) D-amino acid oxidase - termed mDAAO - fused to a red fluorescent protein (mCherry) [24] using lentivirus approaches as described previously [27]. Purified lentiviral particles encoding for cytosolic targeted mCherry-mDAAO chimera were used to infect endothelial cells. After fluorescence-activated cell sorting (FACS), correct targeting and robust expression levels have been validated with high-resolution live-cell epifluorescence imaging. After FACS, the stable cell line displayed 100% transfection efficiency, documented by high-resolution live-cell imaging experiments in the red channel (Figure 2A).

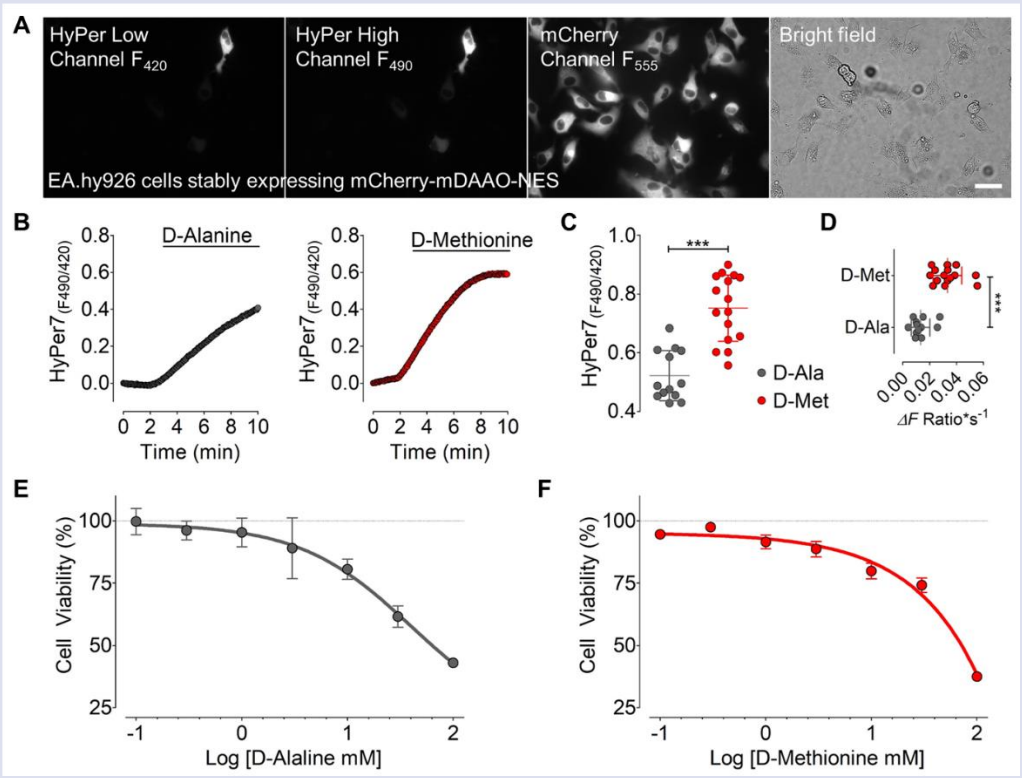

Figure 2: Chemogenetic generation, imaging, and testing of the cytotoxicity of H_2O_2 in endothelial cells. (A) Representative widefield images show EA.hy926 cells transiently expressing HyPer7 biosensor excited with 420 nm (1st image) and 490 nm (2nd image). The emission in both images was collected at 520 nm. The third image shows the same cells in the RFP channel excited with 555 nm. The very right panel shows brightfield images of the same cells. Micrographs are representative, and experiments were repeated n=8 with similar results. (B) Real-time traces of HyPer7 ratio signals of cells stably expressing mCherry-mDAAO-NES that are transiently transfected with HyPer7-NES in response to 10 mM D-alanine (left panel, n=13) and 10 mM D-methionine (right panel, n=16). (C) Scatter dot plot shows maximum HyPer7 ratio amplitudes in response to 10 mM D-alanine (grey dots, n=13) and 10 mM D-methionine (red dots, n=16). (D) Scatter dot plot shows the initial cytosolic invasion of H_2O_2 in the same cells described in panels B-C. (E, F) The normalized curves show cell viability of EA.hy926 cells stably expressing mCherry-mDAAO-NES using MTT assay in response to different D-alanine concentrations (grey dots, n=3) or D-methionine (red dots, n=3). Cells have been treated for 24 hours with 0 mM, 0.1 mM, 0.3 mM, 1mM, 3mM, 10mM, 30mM, and 100mM D-amino acids. An unpaired Student's t-test was applied to compare two columns. (P-value < 0.0001) All values are given as SD.

mDAAO functionality test has been validated with the ultrasensitive H_2O_2 biosensor HyPer7. HyPer7 biosensor is a ratiometric probe permitting the quantification of H_2O_2 levels [15]. Furthermore, these biosensors can be differentially targeted to specific subcellular locales allowing simultaneous detection of the chemogenetically generated H_2O_2. For this purpose, EA.hy926 cells stably expressing mCherry-mDAAO-NES were transiently

transfected using conventional transfection reagents. As shown in Figure 2A, transient transfection of EA.hy926 cells yielded only a few positively HyPer7 transfected cells, which is in line with numerous studies that document the hard transfectability of vascular cells [27,29,31,32]. Because this experiment aims to validate the functionality of mDAAOs in endothelial cells, the number of positively transfected HyPer7 cells (13,3% ± 1,8) was considered sufficient.

Most studies that employed mDAAOs utilized D-alanine as a substrate to activate the chemogenetic enzyme for H_2O_2 production [33]. D-alanine proved suitable as it causes robust and rapid H_2O_2 generation in most cell types, probably due to L-amino acid transporters' (LATs) abundant expression [34]. Luckily, amino acid transporters are selective for their respective amino acid, yet they are less stereoselective, permitting cellular uptake of D-amino acids and the activation of the chemogenetic enzyme [35].

As shown in Figure 2B, administration of 10 mM D-alanine to endothelial cells co-expressing targeted mCherry-mDAAO and HyPer7 triggered a detectable yet sluggish H_2O_2 signal in the cell cytosol. Our group's previous study showed that 10 mM D-alanine triggered robust and fast H_2O_2 generation in different cell types (i.e., HEK293T or U87MG) [24]. This observation suggests that endothelial cells hardly take up D-alanine, probably due to a tightly regulated membrane trafficking and import/export mechanisms in these cell types. Notably, endothelial cells have a barrier function to essential organs, such as the blood-brain, blood-retinal, gut-vascular, or blood-bile-barrier. Thus, the inner layer of vessels plays a critical role as a gatekeeper, firmly regulating the entry of biomolecules [36]. Thus, in contrast to most cell lines (mainly cancer cells), limited D-alanine uptake in endothelial cells might be causative for reduced chemogenetic generation of H_2O_2.

In contrast, D-methionine displayed a significantly higher HyPer7 ratio amplitude in EA.hy926 cells expressing mCherry-mDAAO-NES. However, it is unclear whether endothelial cells favor D-methionine over D-alanine or whether mDAAOs have higher catalytic activity for D-methionine. A likely effect is that both scenarios are valid, contributing to faster and more H_2O_2 generation in these cell types in response to D-methionine.

Nevertheless, both D-amino acids triggered cell death in endothelial cells equally. Cell treatment with up to 1 mM D-amino acid for 24 hours did not significantly affect cell death, while 10 mM and higher concentrations heavily influenced cell death. It is noteworthy that these findings demonstrate the robustness of endothelial cells against sustained oxidative stress that have been exposed to high levels of cytosolic H_2O_2 over 24 hours. Strikingly, only at maximum enzymatic (mDAAO) capacity activated by <10 mM D-alanine or D-methionine caused significant cell death.

Notably, this study focused on cytosolic oxidative stress, a cell compartment decorated with powerful antioxidant systems [37]. Employing the experimental approaches in different cellular locales (i.e., mitochondria, caveolae, nucleus, mitochondria-associated membranes (MAMs)) might shed light on ultra-locale oxidative stress and its role in pathophysiology and cell death in the future.

Conclusion

This study demonstrates a reliable method for manipulating oxidative stress levels in cellular compartments with high spatial and temporal resolution in hardly transfectable endothelial cells. The choice of D-amino acids plays an essential role in modulating kinetic parameters in H_2O_2 generation, which is critical to investigating the acute effects of ROS levels on redox-sensitive enzymes such as the endothelial nitric oxide synthase (eNOS). This study has employed the ultrasensitive H_2O_2 biosensor HyPer7 as a direct read-out for H_2O_2 -generation. Alternative biosensors such as geNOps for nitric oxide (NO) [38] , GCaMPs [39] for calcium, and MALions [40] for ATP-imaging can be combined with chemogenetic approaches presented in this study to investigate the acute or long-term effects of ultra-local H_2O_2 with differentially targeted mDAAOs. Notably, these signaling and metabolic parameters are hallmarks of most vascular-derived diseases.

Acknowledgments

This research was supported by funds from the Scientific and Technological Research Council of Turkey Grant 118C242. The author acknowledges Melike Seçilmiş for her technical support in culturing cells, Tuba Akgül Çağlar for Turkish language editing, Zeynep Çokluk for providing MTT protocols, and Asal Ghaffari Zaki for English language proof-reading. The HyPer7 biosensor was generously provided by Vsevolod Belousov. Figure 1 was generated with Biorender (Licence code: DN23VJT82I).

Conflicts of interest

There are no conflicts of interest in this work.

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