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Dissecting *in vivo* and *in vitro* redox responses using chemogenetics

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Abstract

Hydrogen peroxide (H_2O_2) is the most abundant reactive oxygen species (ROS) within mammalian cells. At low concentrations, H_2O_2 serves as a versatile cell signaling molecule that mediates vital physiological functions. Yet at higher concentrations, H_2O_2 can be a toxic molecule by promoting pathological oxidative stress in cells and tissues. Within normal cells, H_2O_2 is differentially distributed in a variety of subcellular locales. Moreover, many redox-active enzymes and their substrates are themselves differentially distributed within cells. Numerous reports have described the biological and biochemical consequences of adding exogenous H_2O_2 to cultured cells and tissues, but many of these observations are difficult to interpret: the effects of exogenous H_2O_2 do not necessarily replicate the cellular responses to endogenous H_2O_2. In recent years, chemogenetic approaches have been developed to dynamically regulate the abundance of H_2O_2 in specific subcellular locales. Chemogenetic approaches have been applied in multiple experimental systems, ranging from *in vitro* studies on the intracellular transport and metabolism of H_2O_2, all the way to *in vivo* studies that generate oxidative stress in specific organs in living animals. These chemogenetic approaches have exploited a yeast-derived D-amino acid oxidase (DAAO) that synthesizes H_2O_2 only in the presence of its D-amino acid substrate. DAAO can be targeted to various subcellular locales, and can be dynamically activated by the addition or withdrawal of its D-amino acid substrate. In addition, recent advances in the development of highly sensitive genetically encoded H_2O_2 biosensors are providing a better understanding of both physiological and pathological oxidative pathways. This review highlights several applications of DAAO as a

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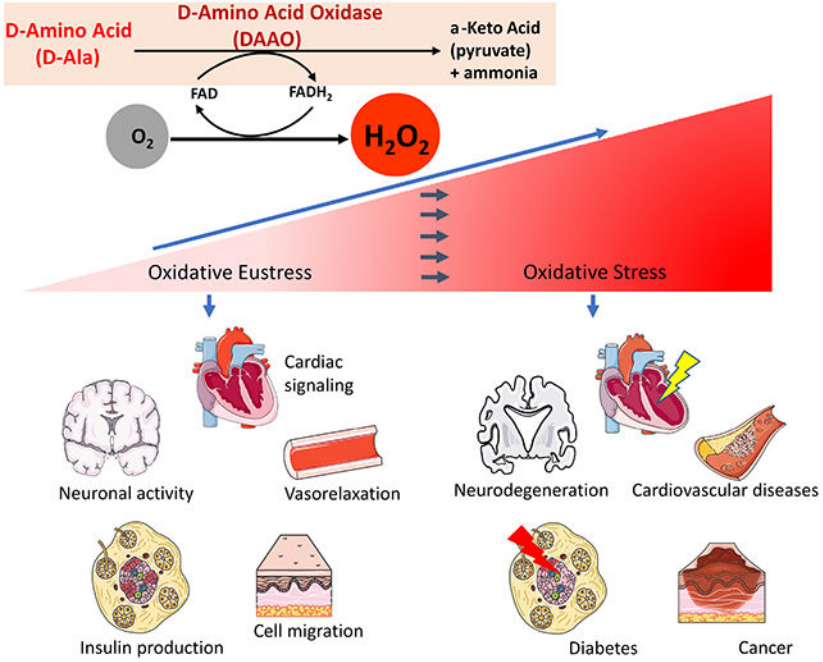
chemogenetic tool across a wide range of biological systems, from analyses of subcellular H_2O_2 metabolism in cells to the development of new disease models caused by oxidative stress *in vivo.*

Graphical Abstract



Keywords

D-amino acid oxidase; Hydrogen peroxide; ROS signaling; Oxidative stress

1. The maintenance of the intracellular hydrogen peroxide balance

Reactive oxygen species (ROS) is a term that has been used to collectively describe the various O_2-derived molecules that can arise within cells. Numerous reviews have described the cellular metabolism of ROS in detail [1-22]; this review will focus primarily on the stable ROS hydrogen peroxide (H_2O_2). It is very important to note that the various ROS are not created equal, either in terms of their chemical reactivities, cellular roles, or subcellular localizations. Some ROS–including hydroxyl ($HO^{\bullet}$), peroxyl ($ROO^{\bullet}$) or alkoxyl ($RO^{\bullet}$) radicals– which can form as by-products of cellular oxidative reactions– are so highly reactive that they are quite unlikely to subserve any physiological signaling role. Since no specific cellular defensive mechanisms exist for their inactivation, these highly reactive ROS can indiscriminately damage nearby biomolecules, leading to deleterious effects on cell function. The superoxide anion ($O_2^{\bullet-}$) is less reactive than the hydroxyl, peroxyl or alkoxyl radicals. $O_2^{\bullet-}$ can be generated in mitochondria by the electron transport chain, and can also be synthesized in various subcellular locales by a family of membrane-bound NADPH oxidases (NOX). Other intracellular enzymes can generate $O_2^{\bullet-}$, including xanthine oxidases, nitric oxide synthases, and cytochrome p450 monooxygenases. Under most cellular conditions, $O_2^{\bullet-}$ is rapidly converted to hydrogen peroxide (H_2O_2) via

mitochondrial or cytosolic superoxide dismutases. Under conditions of oxidative stress, $O_2^{\bullet -}$ can also react with iron and form the highly reactive hydroxyl radical ($HO^{\bullet}$) via the Fenton or the Haber-Weiss reactions [1,2]. Both because of its negative charge and its high reactivity, $O_2^{\bullet -}$ is less likely to serve as a physiological cellular signaling molecule. The stable ROS H_2O_2 is recognized as the principal intracellular species responsible for redox signaling. H_2O_2 mediates a variety of crucial biological functions such as cell differentiation, proliferation, and migration in normal cells, yet at higher concentrations H_2O_2 also can cause pathological oxidative stress [13,16]. Physiological roles for the various free radical ROS have been more difficult to establish, and higher ROS concentrations have been implicated in multiple disease states including cardiovascular and neurodegenerative disorders, chronic inflammatory disease [17,18], cancer, or the aging process itself [19]. The pathophysiological progression of such diseases can be prevented or accelerated by oxidative influences from the environmental factors of the so-called “exposome”, which integrates the effects on oxidative metabolism of lifestyle, nutrition [20], and physical activity [21].

This review will focus on the intracellular metabolism of H_2O_2, both as an endogenous biomolecule in mammalian cells and as a product of the chemogenetic recombinant enzyme D-amino acid oxidase (DAAO). H_2O_2 is differentially distributed within cells, with highest H_2O_2 concentrations found in the peroxisomes and the endoplasmic reticulum (ER). Peroxisomes may serve as sinks for H_2O_2 [15,22] while the high H_2O_2 levels found in the ER might be required for redox-modulated protein folding and bridging disulfide bonds among cysteine residues [4]. In most cells, the overall intracellular H_2O_2 concentration is maintained in the low nM range by the balance of oxidative and reductive enzyme pathways [3,5]. In order to maintain this balance of H_2O_2, different intracellular organelles contain a distinct combination of redox-active enzymes including catalases, peroxiredoxins, thioredoxin reductases, glutathione peroxidases and reductases as well as the small redox protein thioredoxin and the antioxidative tripeptide glutathione [21]. On the one hand, this differential distribution of redox enzymes and their substrates may allow the cell to selectively counteract oxidative stress in different organelles. On the other hand, the differential subcellular distribution of enzymes involved in H_2O_2 metabolism facilitates dynamic physiological ROS signaling responses to discrete cell stimuli [16]. In addition, the transport of H_2O_2 between different subcellular organelles is itself dynamically regulated by peroxiporins, which belong to the family of aquaporin H_2O transporters. Peroxiporins are differentially expressed in tissues and within cells and facilitate the transfer of H_2O_2 through cellular membranes and participate in physiological H_2O_2 homeostasis as well as in oxidative stress (see section 6) [6-8]. The wider view of redox signaling has been reviewed extensively, and will not be presented here in detail [3,9-12,14,16]. However, considering the complexity of intracellular H_2O_2 generation, degradation, and inter-organelle fluxes, it is challenging to dissect the various oxidative pathways within different cells and tissues. Moreover, the detection limits of many biosensor systems have confounded efforts to sensitively and specifically quantitate intracellular H_2O_2 levels in living cells, and the manifold roles of H_2O_2 in oxidative eustress and distress remain incompletely understood [15]. This review discusses the development and cellular applications of a new and promising chemogenetic approach that utilizes a H_2O_2-generating enzyme from yeast,

D-amino acid oxidase (DAAO). Chemogenetic approaches using DAAO are successfully being used to dissect and decipher the complex pathways of ROS metabolism in health and disease.

2. DAAO: an old enzyme goes chemogenetic

D-amino acid oxidases (DAAO) exist as distinct highly stereoselective enzyme isoforms within eukaryotic organisms. The DAAO enzyme and its cofactor flavin adenine dinucleotide (FAD) were first discovered by the metabolic pioneers Hans Adolf Krebs [23] and Otto Warburg [24] in 1935 and 1938, respectively. Over the subsequent decades, the physiological functions of DAAO were identified and described in a broad variety of organisms, from yeast to humans, whereas plants mostly lack this enzyme [25,26]. Different DAAO isoforms possess specific substrate-binding domains that catalyze the oxidation of D- but not L-amino acids with exquisite stereospecificity in the presence of the cofactor FAD. The oxidative deamination of the D-amino acid results in the generation of its corresponding α-keto acid, plus equimolar quantities of ammonia and H_2O_2 (Figure 1). In mammalian cells, the DAAO co-products ammonia plus α-keto acid are usually present in much higher intracellular concentrations than H_2O_2 [27], so there is only a nominal change in intracellular ammonia and keto acid levels, whereas H_2O_2 levels may increase markedly from their typically low basal levels following DAAO activation in cells [27,28].

Much of what has been learned about DAAO catalysis derives from the work of Pollegioni and colleagues, who have characterized in detail the specificity of D-amino acids for DAAO from various species, especially those in humans and of the yeast *Rhodotorula gracilis* [25,29-34]. In humans, DAAO and D-amino acids are primarily present in selected regions of the brain and in some nerves, with a much more limited distribution in other tissues. However, all mammalian DAAO isoforms appear to have relatively low enzyme activity, and are predominantly localized in peroxisomes due to the presence of a peroxisomal targeting sequence; the generated H_2O_2 gets rapidly and effectively eliminated by catalase, which is co-localized in the mammalian peroxisome [29,33]. Thus, under physiological conditions in mammalian cells, endogenous DAAO-generated H_2O_2 accounts for minimal cytotoxicity even in the presence of D-amino acids. In specific regions of the mammalian brain, neuronal L-serine can be converted by the serine racemase to generate D-serine [35]. D-serine can then activate synaptic N-methyl-D-aspartate receptors (NMDAR), which may have a neuroprotective role mediating physiological effects [36-38]. In some subsets of neurons, it appears that the function of peroxisomal DAAO in neurons is to modulate the intracellular pool of D-amino acids, in particular D-serine. In selected regions of the brain, DAAO mutants lacking the enzyme's peroxisomal targeting sequence can lead to a decrease in neuronal D-serine, which has been implicated in development of neuropathologic states such as schizophrenia [39].

In contrast to these very limited roles for DAAO in most mammalian cells, DAAO in yeast actually represents a key enzyme that plays a central role in energy metabolism. In yeast, D-amino acids are used as sources of carbon, nitrogen, and energy [40-42]. In contrast to the catalytic properties of mammalian DAAO, yeast DAAO exhibits a 400-fold higher flavin binding affinity [43,44]. Analyses of the crystal structure of mammalian and yeast DAAO

reveals that for both enzymes, the apoenzyme is a monomer that forms active dimers upon binding FAD, which then binds and oxidizes the substrate D-amino acid [45-48]. FAD may be a rate limiting factor for the DAAO enzymatic reaction, especially when the recombinant enzyme is used in cultured cells. Indeed, it has been reported that the FAD concentration in many tissues can be orders of magnitude higher than the FAD levels found in cultured cells [49,50]. Thus, the supplementation of > 2.5 μM FAD has been recommended for chemogenetic *in vitro* approaches in cultured cells expressing recombinant yeast DAAO [51]. Moreover, the binding capacity of D-amino acids for mammalian DAAO is lower than in yeast DAAO since the yeast enzyme does not possess an inhibitory active site "lid" that is present in mammalian DAAO variants. Thus, the rate of catalytic conversion of D-alanine to H_2O_2 by yeast DAAO is more than 30-fold higher compared to mammalian DAAO [52,53]. The substrate selectivities of D-amino acids have been screened for several yeast DAAO isoforms, revealing a K_m of 1 μM for D-alanine [54,55]; yeast DAAOs showed higher rates of H_2O_2 production when D-alanine was the substrate, as compared to D-serine [56]. Taken together the mechanistic and kinetic properties of yeast DAAO make it much more efficient at the generation of H_2O_2 compared to mammalian DAAO; indeed yeast DAAO has been used as a biotechnological tool for the synthesis of cephalosporin antibiotics [57-59]. *Unless otherwise noted in this review, the term DAAO refers to the yeast enzyme* [43].

The term 'chemogenetic' has been used to describe model systems where recombinant proteins get activated by a highly specific and unique ligand or substrate [60]. Chemogenetic approaches were used to develop *"designer" receptors activated solely by a synthetic ligand* (RASSLs), which now are more commonly termed *designer receptors exclusively activated by designer drugs* (DREADDs) [61,62]. DREADDs have been principally used to study G protein-coupled receptors in the nervous system [63,64] and enable an "on/off" receptor activation upon application of specific chemically engineered molecules or ligands. Chemogenetic approaches have also been developed using an enzyme's unique substrate instead of a receptor's unique ligand. DAAO represents a chemogenetic enzyme that generates H_2O_2 only in the presence of its substrate D-amino acids. Beside DAAO, also other enzymatic models for intracellular H_2O_2 production have been developed and utilized in the past such as uricase [65], glucose oxidase [66] tyrosinase [67], glycolate oxidase [68], alcohol oxidase [69], L-amino acid oxidase [70], the NADPH dependent cytochrome P450 BM3 [71] as well as light-activated probes [72,73]. But in contrast to the chemogenetic approach using DAAO and its unique D-amino acid substrate, these other enzymes can act on endogenous substrates, making it difficult to dynamically regulate intracellular H_2O_2 generation due to presence of endogenous substrates. Therefore, researchers developed a chemogenetic approach based on DAAO to specifically and dynamically modulate intracellular H_2O_2 mediated pathways.

One of several biological applications for DAAO is in the calibration and validation of H_2O_2 detection systems as well as for studying intra-organelle diffusion of H_2O_2 [74-77]. Although several chemical probes, including the redox-sensitive dye dichlorofluorescine (DCF) [78], have been used to measure H_2O_2 [79], these dyes lack specificity for H_2O_2 as they can easily be oxidized by other cellular oxidants [80] or may even generate ROS such as $O_2^{\bullet -}$ [81]. In contrast, the development of genetically encoded fluorescent indicators has enabled more specific and sensitive detection of H_2O_2. Although some fluorophores

can be pH-sensitive [82], most genetically-encoded biosensors provide several advantages in live cell imaging. The commonly-used H_2O_2 biosensors detect H_2O_2 as consequence of the H_2O_2-dependent oxidation of vicinal cysteine thiols leading to the formation of a disulfide bond, which results in a conformational change within the sensor protein and a change in its fluorescent properties. For instance, introducing cysteines at distinct positions of a green fluorescent protein (GFP) yielded the redox sensitive biosensor, roGFP [83]. The fluorescent biosensor termed "HyPer" is composed of a circularly permutated yellow fluorescent protein N- and C-terminally flanked by the split sensory elements of the H_2O_2 dependent transcription factor OxyR from *E. coli* [84]. Ratiometric H_2O_2 indicators based on Foerster resonance energy transfer (FRET) have also been developed in which a H_2O_2 sensitive peptide is inserted between two different fluorescent proteins [85,86]. More recently, several of these genetically-encoded biosensors have been modified, leading to enhanced H_2O_2 sensitivity [87-89]. In particular, a novel FRET-based sensor has been developed based on peroxiredoxin-2 [90]; in addition the newest generation of Hyper sensors, HyPer7 uses the OxyR sequence from *Neisseria meningitidis* [88], providing enhanced H_2O_2 sensitivity in the low nanomolar range and also showing improved pH stability.

In contrast to most of the chemical dyes that detect intracellular biomolecules, genetically-encoded biosensors can be differentially targeted to different regions within cells, permitting the detection of H_2O_2 in specific subcellular organelles. DAAO can also be expressed as a fusion protein with HyPer, enabling the generation and detection of H_2O_2 by a single protein. The first study using HyPer fused with DAAO validated the kinetics of DAAO-mediated H_2O_2 generation, and demonstrated this production to be dependent on and titratable to the D-alanine concentration provided to the cell [91]. By using a cardiomyocyte-specific promoter the same construct has been successfully applied *in vivo* to study the effects of oxidative stress in rat hearts (see section 4) [92]. In separate studies, DAAO was fused with an ultrasensitive FRET based H_2O_2 probe [90] or with a genetically-encoded NADPH indicator to study the effect of oxidative stress on the mitochondrial NADPH pool [93]. The development of novel genetically-encoded H_2O_2 biosensors also revealed the existence of a large H_2O_2 gradient across the plasma membrane, showing that the ratio between extracellular and intracellular H_2O_2 levels was ~400 to 1 [88,94]. Indeed, plasma levels of H_2O_2 were also found in the micromolar range– orders of magnitude higher than those previously estimated to be present inside cells [9,95]. Since conventional experimental approaches using extracellular administration of H_2O_2 need to overcome cellular H_2O_2 gradient to study oxidative effects within cells, the high concentrations of extracellular H_2O_2 can result in spurious cellular responses, and should be interpreted with caution [96,97]. Instead, the applications of the DAAO system provide a more precise method to mimic endogenous H_2O_2 mediated pathways in oxidative eustress or distress deriving from distinct intracellular sources [98,99] as summarized below and as shown in Figure 2.

3. Chemogenetic H_2O_2-generating approaches in vascular cells

The vascular system dynamically regulates blood flow according to the diverse metabolic needs of different tissues [100]. The endothelial layer of the vascular system is critical in regulating this homeostatic system [101]. Oxidative stress is associated with endothelial dysfunction and with pathophysiological conditions such as hypertension [102,103],

vascular inflammation [104,105], diabetes [106] and adverse vascular remodeling [107-110]. Yet H_2O_2 also has an essential role as a signaling molecule in the vascular endothelial cell to maintain this vascular homeostasis through involvement in post-translational modifications such as phosphorylation [106,111-114], S-glutathionylation [115-118], sulfenylation (RSOH) [119,120] and other cysteine modification (cysteinylation and disulfide bond formation) [121,122]. It is critical to regulate the optimal amount of H_2O_2 in the endothelial cells to mediate these diverse oxidant-dependent post-translational signaling responses. Perturbed H_2O_2 homeostasis leads to impaired cell function, increasing the risk of disease-associated pathologies [108,123,124]. One important issue in understanding this homeostasis is determining the actual concentrations of H_2O_2 at physiological levels in different compartments of the endothelial cell. The recently developed Hyper7 genetically-encoded biosensor [88], is an ultrasensitive probe for the detection of H_2O_2, and can be combined with DAAO to modulate H_2O_2 levels in different organelles in vascular endothelial cells [98]. Using this chemogenetic approach along with live-cell imaging, H_2O_2 can both be endogenously produced and detected in an organelle-specific manner in the cytoplasm, caveolae, mitochondria, or nucleus. Saravi et al. have recently shown that compartmentalized endogenous generation of H_2O_2 elicits differential signaling effects (through altered Akt, AMPK and eNOS phosphorylation) compared to traditional extracellular treatment of H_2O_2 in endothelial cells even though levels of H_2O_2 achieved by both methods were quantitatively similar in the cells [98]. They also found that AMPK phosphorylation directly depends on nuclear H_2O_2. The inhibition of AMPK phosphorylation diminished nuclear H_2O_2 driven eNOS phosphorylation but had no effect on cytosol- or caveolae-generated H_2O_2 mediated phosphorylation response. This clearly suggests that compartmentalized H_2O_2 is critically essential for signaling events in endothelial cells.

Thus, spatiotemporal tracking of H_2O_2 levels in living cells is crucial for understanding the role of this redox molecule in vascular biology. A chemogenetic approach can be an invaluable tool for the study of vascular dysfunction caused by disturbance of H_2O_2 homeostasis. The opportunities that lie ahead with the newly developed HyPer7-DAAO sensors may be equally promising and should lead to a much better understanding of tissue-, cell- and subcellular-specific redox homeostasis.

4. A heart failure model based on DAAO induced oxidative stress

Oxidative stress has been implicated in the pathogenesis of heart failure and in many cardiac disease states [125]. Although these associations have been well described, it is still not clear if oxidative stress alone is a causal factor of heart failure. First insight into this question was recently provided by the application of chemogenetic approaches *in vivo.* Specifically, a new model of heart failure was developed by using DAAO to generate the stable ROS H_2O_2 in cardiac myocytes in living rats [92,126]. The DAAO construct was packaged as a fusion protein with the HyPer biosensor in the cardiotropic adeno-associated virus isotype 9 (AAV9) under control of the cardiac-specific cTnT promoter [92]. The animals were then infected with the recombinant DAAO-AAV9 virus by tail vein injection, and robust expression in the cardiac myocytes was detected within 3–4 weeks. The expression was specific to the heart, with only minimal expression in skeletal muscle [92]. Importantly,

when D-alanine was added to isolated adult cardiac myocytes, there was robust generation of H_2O_2, detected using the HyPer biosensor. Of note, the addition of L-alanine did not result in H_2O_2 generation: most mammalian cells use L-amino acids and the recombinant DAAO is inactive until D-amino acids are provided [126].

To further investigate the effects of chronic oxidative stress in cardiac myocytes, animals were infected with the DAAO-AAV9 and were provided with D-alanine in their drinking water. This led to a striking dilated cardiomyopathy phenotype within 4 weeks of D-alanine treatment, characterized by significant reductions in left ventricular (LV) ejection fraction and fractional shortening along with significant increases in LV volume and heart mass [92,126]. The hemodynamic changes were accompanied by significant increases of the biomarkers atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and cardiac troponin I (cTnI) both in the plasma and the cardiac transcriptome as has been similarly reported in humans with heart failure [92,126,127]. Despite the decline in cardiac function, there was no evidence of interstitial fibrosis by 4 weeks of D-alanine feeding. With longer provision of D-alanine, there was no further decline in cardiac function, but there was development of cardiac fibrosis at 8 weeks as seen by increased collagen deposition on histology and elevation of several transcriptional markers of fibrosis including mRNAs encoding selected collagen isoforms and transforming growth factor-β1. The robust heart failure phenotype was rapidly reversed when DAAO infected animals were treated with angiotensin-II receptor blocker valsartan alone or in combination with the neprilysin inhibitor sacubitril even in the presence of ongoing oxidative stress [126]. Interestingly, despite the recovery of cardiac function with drug treatment, the interstitial fibrosis persisted. These findings indicate that cardiac fibrosis and cardiac dysfunction should not be used interchangeably as the presence of one does not necessarily imply the presence of the other.

In conclusion, the DAAO model of chemogenetic heart failure induces a rapid, robust and tractable heart failure phenotype that is reversible and has many features in common with human heart failure. This chemogenetic approach adds new insights and establishes oxidative stress as a causal determinant of heart failure. This new model may now be utilized for the discovery and validation of new therapeutic targets for the prevention and treatment of heart failure.

5. Using DAAO in a neurobiological system

In contrast to the deleterious effects of chemogenetic oxidative stress on cardiac myocyte function [92], in other model systems the chemogenetic generation of lower H_2O_2 concentrations can have cytoprotective effects. ROS imbalance is also a major cause of neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and amyotrophic lateral sclerosis [128]. The molecular mechanisms, whereby alterations in redox balance lead to neurodegeneration, are incompletely understood, but several reports have implicated a neuroprotective role of the redox-modulated transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) [129-131]. However, a study using cytosolic DAAO expression in astrocytes co-cultured with neurons successfully led to a redox-regulated neuroprotection that was independent of Nrf2 [132]. Astrocytes and neurons

are tightly interconnected via gap junctions to ensure crucial cell communication between the two neuronal cell types [133,134]. The exposure of DAAO-expressing astrocytes to high concentrations of D-alanine resulted in neurotoxicity, but the application of a lower D-alanine concentration enabled H_2O_2 transfer to neurons that led to the inhibition of protein tyrosine phosphatase within the astrocytes. Microarray analyses of the axonal transcriptome revealed an up-regulation of Interleukin-1beta (IL-1β) and Caveolin-1, but no increased gene expression of Nrf2-regulated genes. The authors could mimic the neuroprotective H_2O_2-induced effect with PTP inhibitors, indicating a possible neuroprotective mechanism in response to IL-1β upregulation [135]. In contrast, a subsequent publication reported that extracellular H_2O_2 promoted activation of Nrf2 in astrocytes under the same conditions [136]. These disparate findings again point to the divergent responses that can be elicited depending on the cellular source of H_2O_2 [137]. Despite the long-held view that neurons are particularly vulnerable to oxidative stress [138,139], these studies show that H_2O_2 can be involved in both physiological and pathological responses in neurons. Moreover, the work of Haskew-Layton et al. to date represents the only investigation in which DAAO-mediated H_2O_2 generation is used to elicit a physiological instead of a pathological response.

6. Visualizing inter-organelle H_2O_2 transfer with HyPer and DAAO in a diabetic model

Pancreatic β-cells, which produce and secrete insulin, are the largest cell population of the islets of Langerhans and are particularly vulnerable to oxidative stress [140]. This exceptional sensitivity is due to the fact that β-cells typically express low levels of antioxidant enzymes [141,142]. Catalase is almost completely absent in the peroxisomes of β-cells, and glutathione peroxidases in the mitochondria and ER are only weakly expressed [143,144]. On the other hand, β-cells generate considerable amounts of H_2O_2 as a by-product of disulfide formation during the synthesis of insulin [145]. Production of H_2O_2 is further enhanced in pathologic states. During cytokine attack by immune cells in type 1 diabetes mellitus (T1D), large amounts of H_2O_2 are produced in the mitochondria [146-148]. In the pathogenesis of type 2 diabetes mellitus (T2D), H_2O_2 is formed from the peroxisomal β-oxidation of long-chain free fatty acids [149]. Thus, increased ROS production can lead to the development of diabetes, as H_2O_2 cannot be sufficiently detoxified. It is therefore even more important that H_2O_2 can be transported to a site with a higher antioxidant capacity. Otherwise, toxic $OH^{\bullet}$ may arise in the Fenton reaction, which can lead to β-cell apoptosis [150,151]. Differentially-targeted DAAO constructs (targeted to mitochondria, peroxisomes, and/or ER)– in combination with targeted HyPer constructs– allow monitoring of H_2O_2 transport across membranes. By expressing DAAO in the mitochondria and HyPer in the peroxisomes, it is possible to follow the path of H_2O_2 across two membranes from one organelle to another. This approach is used in research on peroxiporins, which are a class of aquaporins that transport not only water but also H_2O_2. The various peroxiporin isoforms have different H_2O_2 transport capacities [76,152-157]. Recombinant DAAO/HyPer approaches have been used to explore the localization and functionality of different peroxiporins by “knocking out” and/or overexpressing these peroxiporins and then using live cell imaging to quantitate H_2O_2 transfer rates within the cell. For example, one study documented a critical role of the peroxiporin isoform AQP8

in modulating the intracellular distribution of H_2O_2 in insulin-producing β-cells, which are particularly susceptible to oxidative stress [75], and indicating a potential role for AQP8 paticularly in T1D pathogenesis.

7. Targeting cancer cells with DAAO

Excessive H_2O_2 can lead to pathological oxidative stress and cause cellular apoptosis [158,159]. One way to harness this oxidative damage for therapeutic purposes is to specifically target cancer cells for enhanced ROS production in order to induce cancer cell death. Many small molecule redox chemotherapeutics have been investigated for this purpose, but often have shown off-target effects [160]. The use of DAAO as a gene-directed enzyme prodrug represents a potentially effective approach since D-amino acids would not have a cytotoxic effect in cells that are not expressing DAAO. Moreover, some cancer cells appear to be characterized by increased ROS levels [161]. In a recent pioneering study, DAAO was stably expressed in rat 9L gliosarcoma cells, and the addition of D-alanine had a cytotoxic effect [162]. The main drawback of using this chemogenetic approach to kill cancer cells is the reliance on proper targeting of DAAO to tumors *in vivo* since ROS are involved in almost every stage of cancer development [163-165]. To overcome this problem, the conjugation of macromolecules to DAAO has been used to achieve more specific targeting of DAAO to cancer cells; one combination that has been used to accomplish this was polyethylene glycol conjugated (“pegylated”) DAAO (PEG-DAAO). Tumor targeting by pegylation led to the accumulation of PEG-DAAO in solid tumors. Several studies in mice showed enrichment of PEG-DAAO in tumor cells, and the addition of D-proline resulted in marked inhibition of tumor growth as a consequence of H_2O_2 generation– without affecting non-cancerous cells [166]. Moreover, the tumor-killing effects of PEG-DAAO could be improved by the co-application of pegylated zinc protoporphyrin (PEG-ZnPP), a potent heme oxygenase-1 (HO-1) inhibitor [167]. Compared to normal cells, most cancer cells contain a more limited set of antioxidant enzymes [166]. Although these first applications of DAAO for cancer therapy appear promising, the method does have important limitations, e.g. the low O_2 levels in tumor cells led to the development of a DAAO variant that has a higher affinity for molecular oxygen. A mutated variant of a yeast DAAO was developed that showed a lower K_m for D-alanine even under low oxygen condition [168]. Recently, DAAO-mediated chemotherapy has been further developed by coating magnetic nanoparticles with DAAO, enabling the use of an external magnetic field [169-172] to target the enzyme to tumors [173,174]. Sikes and colleagues have established a model in HeLa cells that is based on mitochondria-targeted DAAO as an anti-cancer cell therapy [74,175-178]. The local generation of H_2O_2 by targeting DAAO to mitochondria in HeLa cells showed more cytotoxicity than cytosolic DAAO [74,77,93,99]. Taken together, several approaches using DAAO have been developed to try and target redox-based chemotherapies to tumors, which may have the potential to serve as novel tools to combat cancer [179].

8. Perspectives on the applications of chemogenetics in redox biology

Chemogenetic approaches using DAAO and HyPer represent an example of “synthetic biology”, in which engineering principles are applied to create novel biological systems that

are not typically found in nature. Thus, a yeast enzyme (DAAO) and a bacterial transcription factor (OxyR, which is a key component of HyPer biosensors) have been cloned as a fusion construct and used both to generate (DAAO) and detect (HyPer) H_2O_2 in cultured mammalian cells or in animals. While these approaches are informative as long as proper controls are included (Figure 1), there are potentially important differences between the biological effects of H_2O_2 generated by recombinant DAAO and the H_2O_2 that comes from endogenous enzymes and organelles. These differences are easier to control in the *in vitro* setting, in which real-time imaging of (using HyPer or other biosensors) can be used to dissect intracellular pathways of H_2O_2 diffusion and signaling following the addition (or removal) of D-amino acids. However, the results of these experiments can be influenced by the differential transport of D-amino acids in different organelles and in different cell types [180-183]. Moreover, the rate of reduction of oxidized HyPer might vary in different regions of the cell, and could thereby influence cellular responses. The H_2O_2 generated by, for example, Nox4 in the endoplasmic reticulum, cannot be viewed as being equivalent to the H_2O_2 molecules that are generated by ER-targeted DAAO: there will be differences both in local H_2O_2 flux and in the protein microenvironment that exert influence the cellular response. It can be helpful to use a HyPer control construct (as control for the HyPer-DAAO fusion protein) to avoid potential confounding by HyPer serving as a sink for the H_2O_2 in the cell. In general, however, genetically-encoded biosensors are expressed at a much lower level than the analytes that they detect, so HyPer itself is unlikely to independently change basal intracellular H_2O_2 levels. It is also important to include negative controls that include the use of L-amino acids instead of D-amino acids. All of these considerations also apply in the *in vivo* setting, but once again, confounding can be minimized by the use of proper controls. *In vivo,* the consequences of D-amino acid addition and removal may transpire over a time period of days, and differential D-amino acid uptake between different cell types can also influence the response to chemogenetic H_2O_2 generation. It is important to note that the DAAO-HyPer fusion protein is able to induce chronic oxidative stress *in vivo,* indicating that DAAO generates much more H_2O_2 than HyPer is able to consume [92]. D-amino acid feeding to animals expressing targeted DAAO in different cell types permits the study of oxidative stress in distinct tissues to be correlated with disease phenotype. These *in vivo* approaches can provide important information of the differential roles of specific cells in ROS-dependent disease states. Despite these limitations, properly-designed and carefully-controlled chemogenetic approaches using DAAO can provide novel information on the effects of differentially-generated H_2O_2 both in cultured cells and *in vivo.*

Many decades passed from the discovery of mammalian DAAO in porcine liver to the exploitation of the yeast DAAO as a chemogenetic enzyme to produce H_2O_2 in mammalian cells. Figure 3 shows important milestones on DAAO research from the discovery to its use as a chemogenetic tool [23,24,43,60,92,162,184-190]. At the time of its discovery, the presence of DAAO in mammalian cells seemed highly unusual since D-amino acids are found at very low levels in most mammalian tissues [33]. It has been speculated that mammalian DAAO functions in peroxisomes to catabolize the rare D-amino acids that can found in regions of the brain and in metabolically active tissues such as liver and kidney. While the yeast DAAO follows the same overall catalytic scheme as its mammalian homologues, the yeast enzyme subserves an entirely different physiological role: yeast

DAAO metabolizes D-amino acids to generate energy and provide key metabolites for the cell [41]. The divergent cellular functions of mammalian and yeast DAAO are not only interesting from evolutionary point of view [25], but the distinctively robust generation of H_2O_2 by the yeast enzyme permits the use of yeast DAAO as a chemogenetic tool that permits the modeling of oxidative distress and oxidative eustress. These key features of yeast DAAO have led to exciting new discoveries on the physiological and pathophysiological roles of H_2O_2 in mammalian cells and tissues.

9. Conclusions

H_2O_2 is a key signaling molecule involved in numerous physiological processes in diverse cell types. However, like other ROS, H_2O_2 at high concentrations is deleterious for cells. Over the last century, diseases characterized by excessive ROS accumulation– including diseases of the cardiovascular and nervous system as well as diabetes and cancer– have emerged as the leading causes of death [191]. Chemogenetic approaches using DAAO for H_2O_2 generation allow disease states associated with oxidative stress to be effectively modeled, enabling the more precise understanding of the pathological processes caused by redox stress. To date, relatively few studies have used chemogenetic tools to modulate ROS *in vivo,* but the number of studies utilizing DAAO has been increasing steadily over the last decade (Figure 3). The generation of transgenic animal models with tissue-specific expression of DAAO will enable future investigations of ROS-related pathological processes in diverse tissues and organ systems and lead to the identification of novel therapeutic targets.

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Highlights

- Hydrogen peroxide (H_2O_2) is a key molecule that is involved in both oxidant signaling and oxidative stress.
- D-amino acid oxidase (DAAO) serves as a powerful chemogenetic tool to generate intracellular H_2O_2 and to decipher oxidant-modulated pathways in normal cells and in disease states.
- Chemogenetic applications are increasingly being used to study the roles of H_2O_2 in cardiovascular disease, neurodegeneration, diabetes and cancer.

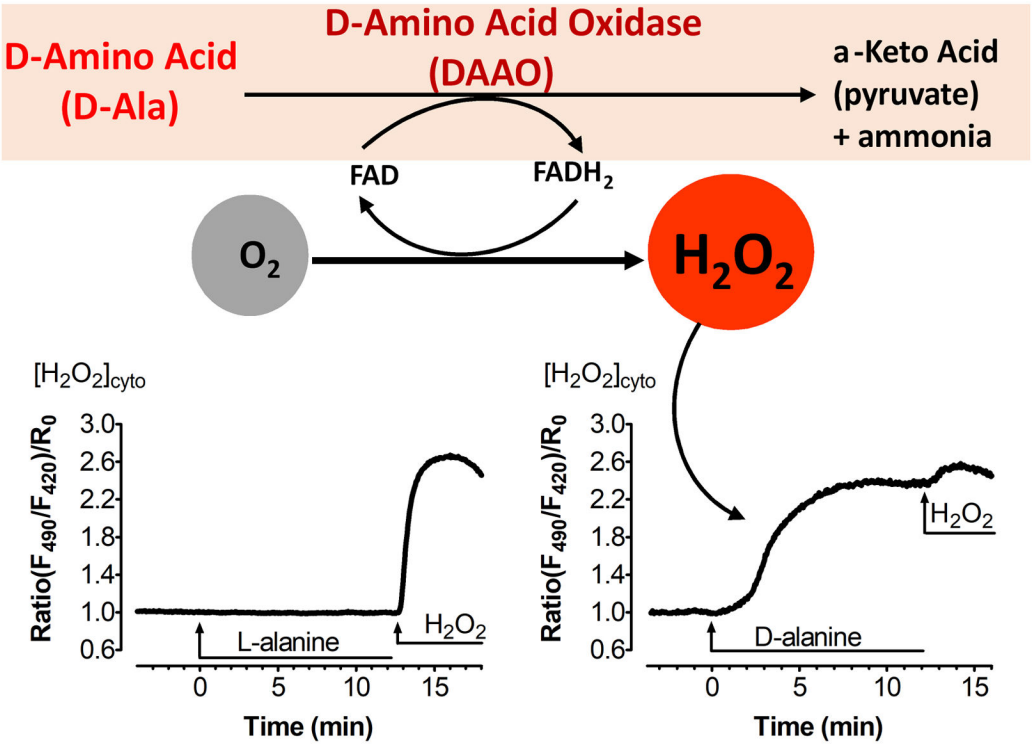

Figure 1: Intracellular H_2O_2 generation by DAAO

This figure shows stereospecific catabolism of D-amino acids to its corresponding α-keto acid, ammonia and equimolar H_2O_2 by DAAO. The enzyme reduces its co-factor FAD to $FADH_2$ and converts molecular oxygen to H_2O_2 (upper panel). Curves are modified from ref. 98, and show H_2O_2 increases in endothelial cells transiently transfected with the DAAO-HyPer and treated with D-alanine (right panel), but not L-alanine (left panel); the subsequent application of extracellular H_2O_2 leads to full oxidation of the HyPer biosensor.

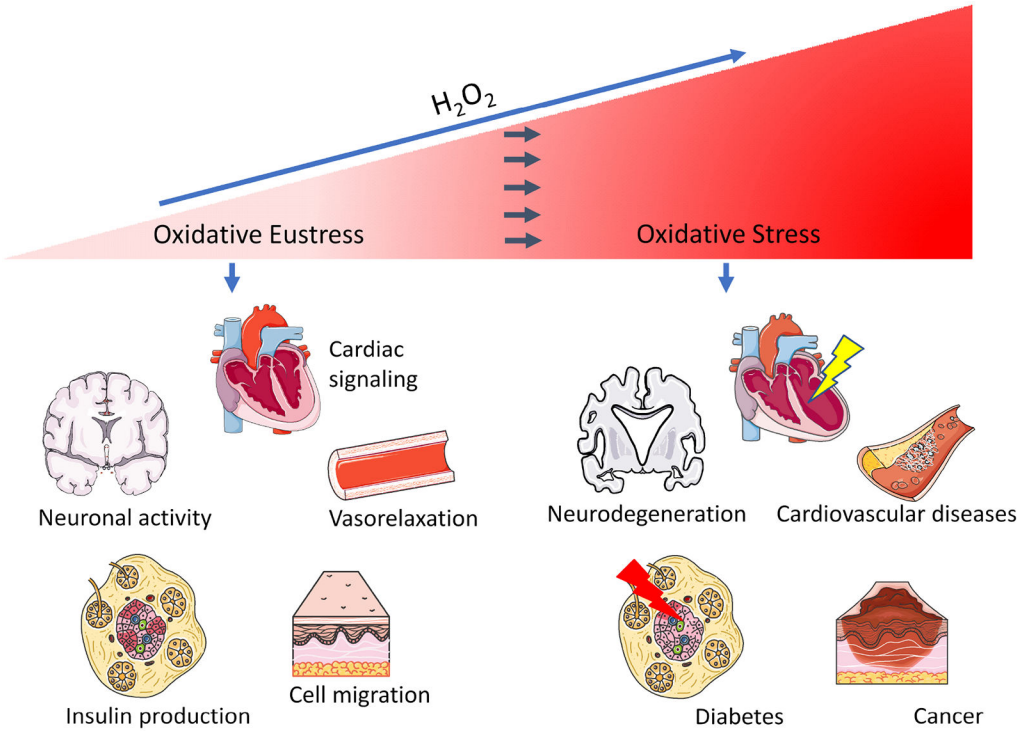

Figure 2: DAAO applications for studying H_2O_2-mediated pathways in various cells and tissues: Scheme shows physiological H_2O_2 signaling in the heart, vasculature, nerves, β-cells and other cells and tissues (left side) or conditions of oxidative stress resulting in cardiovascular disease, neurodegeneration, diabetes and cancer induced by pathologically increased H_2O_2 (right side). Depicted models were modified from Servier Medical Art (https://smart.servier.com/image-set-download/), licenced under a Creative Commons Attribution 3.0 Unported License by Servier.

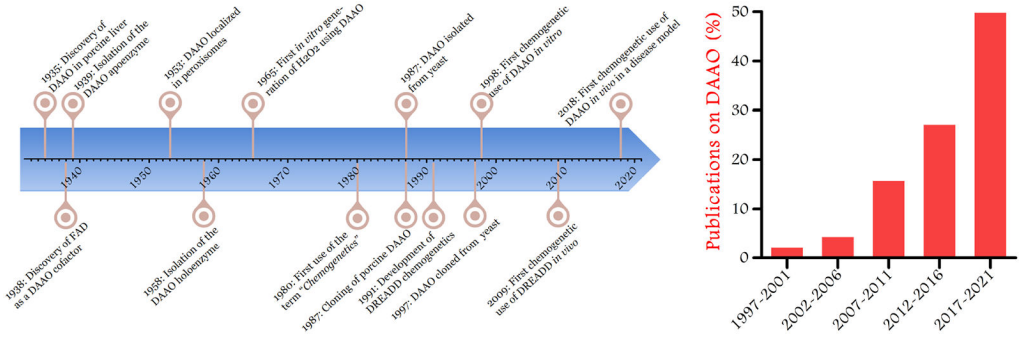

Figure 3: Chronological development of DAAO and chemogenetic research leading to chemogenetic DAAO applications

The timeline shows historical milestones in research on DAAO and chemogenetics (left panel). The graph indicates the percentage of publications using DAAO as a chemogenetic tool for H_2O_2 generation in 5 year intervals (right panel).