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Role of Metabolic H_2O_2 Generation

REDOX SIGNALING AND OXIDATIVE STRESS[*]

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Hydrogen peroxide, the nonradical 2-electron reduction product of oxygen, is a normal aerobic metabolite occurring at about 10 nM intracellular concentration. In liver, it is produced at 50 nmol/min/g of tissue, which is about 2% of total oxygen uptake at steady state. Metabolically generated H_2O_2 emerged from recent research as a central hub in redox signaling and oxidative stress. Upon generation by major sources, the NADPH oxidases or Complex III of the mitochondrial respiratory chain, H_2O_2 is under sophisticated fine control of peroxiredoxins and glutathione peroxidases with their backup systems as well as by catalase. Of note, H_2O_2 is a second messenger in insulin signaling and in several growth factor-induced signaling cascades. H_2O_2 transport across membranes is facilitated by aquaporins, denoted as peroxiporins. Specialized protein cysteines operate as redox switches using H_2O_2 as thiol oxidant, making this reactive oxygen species essential for poising the set point of the redox proteome. Major processes including proliferation, differentiation, tissue repair, inflammation, circadian rhythm, and aging use this low molecular weight oxygen metabolite as signaling compound.

One of the surprises in redox biology was the relatively recent appreciation of hydrogen peroxide as a messenger molecule. It is now widely accepted that this low molecular weight molecule is utilized in metabolic regulation in ways similar to diffusible gases such as NO, CO, or H_2S. Even more so, H_2O_2 is recognized as being in the forefront of transcription-independent signals, in one line with Ca^{2+} and ATP (1). H_2O_2 diffuses through tissues to initiate immediate cellular effects, such as cell shape changes, the formation of functional actomyosin structures, and the recruitment of immune cells (1). Among the various reactive oxygen species, H_2O_2 has been identified as a suitable second messenger molecule, in part because of its reactions with specific oxidation-prone protein cysteinyl residues in local environments that lower the pK_a to provide specificity in time and space, required in signaling (2, 3). However, until recently, assessing the precise amount of hydrogen peroxide in cellular and subcellular locations under *in vivo* conditions was challenging, but promising progress in methodology has opened a new level of analysis, introducing genetically encoded fluorescent indicators as H_2O_2 reporter molecules (4).

Against this background, the present minireview will address the following questions. 1) How can H_2O_2 be assayed in the biological setting? 2) What are the metabolic sources and sinks of H_2O_2? 3) What is the role of H_2O_2 in redox signaling and oxidative stress?

How Can H_2O_2 Be Assayed in the Biological Setting?

In his book "*On the Catalytic Actions of the Living Substance*," in 1928 Otto Warburg (5) noted that one should "study enzymes under the most natural conditions of action, in the living cell itself. From the standpoint of preparative chemistry they may be looked upon as being of utmost impurity. However, if one finds reactants that selectively react with the enzymes, the rest of the cell interacts as little as the glass wall of a test tube in which a chemical reaction is carried out." This is the mindset behind the current use of proteins selectively sensing and reporting ligands or reactants such as H_2O_2.

Organ Spectrophotometry of Catalase Compound I

The first demonstration that H_2O_2 is present as a normal attribute of aerobic metabolism in mammalian cells was by spectrophotometry of catalase Compound I, which is formed in the reaction of catalase with H_2O_2 (6). Catalase *minus* catalase Compound I (7) has an optical difference spectrum in the near infrared amenable to specific spectrophotometry in biological systems because there is negligible interference from other components and little light scattering. The absorbance difference between 640 and 660 nm was identified to selectively monitor the steady-state level of catalase Compound I in intact liver (6), enabling readout of H_2O_2 by using Compound I as a molecular beacon and proving the existence of H_2O_2 under normal metabolic conditions. As illustrated in Fig. 1, the continuous endogenous production of H_2O_2 was demonstrated by its reaction with the hydrogen donor, methanol. There is increased formation of Compound I upon infusion of substrate for enhanced production of H_2O_2, *e.g.* glycolate (8). Methanol can be used as hydrogen donor for titrations in intact tissues because unlike ethanol, it reacts specifically with catalase Compound I. From titrations with methanol, the steady-state rate of H_2O_2 production was quantified to be 50 nmol/min/g of liver, which is about 2% of the respiration rate of the liver (9). Supply of medium-chain fatty acids such as octanoate increased the rate of H_2O_2 generation to 170 nmol/min/g of liver (Table 1). The concentration of H_2O_2 was estimated to be about 10 nM (10). Exposed liver of anesthetized rats *in situ* is amenable to this H_2O_2 assay as well (11). These data represent H_2O_2 detected by catalase in the liver, a tissue rich in peroxisomes (see Ref. 10). Rates and concentrations of H_2O_2 in other cell types may be different. Isolated mitochondria had an upper

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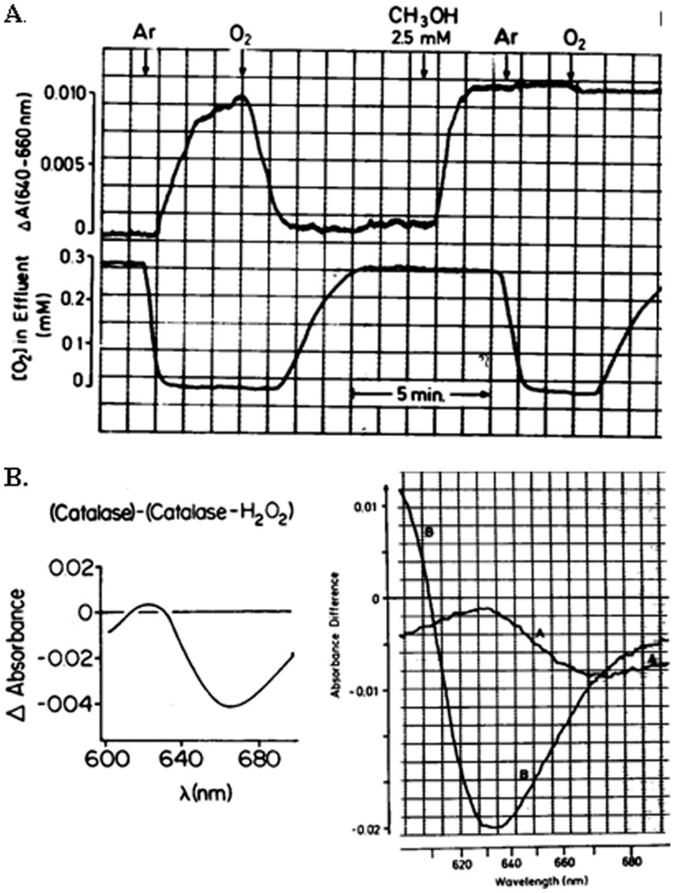

FIGURE 1. **Demonstration of steady-state H_2O_2 generation in intact liver by organ spectrophotometry.** *A*, the absorbance difference between 640 and 660 nm is used for monitoring catalase Compound I (*top*) and oxygen concentration in effluent perfusate (*bottom*). Anoxia and reoxygenation (argon and oxygen, *arrows*) and methanol (*arrow*) as hydrogen donor modulate, and thereby prove the existence of, H_2O_2 steady states; from Sies and Chance (6) with permission. *B*, catalase *minus* catalase Compound I difference spectra. *Left*, isolated enzyme. Right, organ difference spectrum (*trace A*) and cyanide difference spectrum (*trace B*); from Sies *et al.* (8) with permission.

TABLE 1

H_2O_2 production rates in intact organ

Isolated hemoglobin-free perfused liver data were obtained by methanol titration of catalase Compound I; from Oshino *et al.* (9). For discussion, see Refs. 10 and 32.

Substrate or inhibitor	H_2O_2 production rate
	nmol of H_2O_2/min/g of liver wet wt
L-Lactate, 2 mM; pyruvate, 0.3 mM	49
+ Antimycin, 8 μM	75
+ Octanoate, 0.3 mM	170
+ Oleate, 0.1 mM	66
+ Glycolate, 3 mM	490

estimate of the proportion of electron flow giving rise to H_2O_2 with palmitoyl carnitine as substrate of 0.15% (12), an order of magnitude lower than the 2% mentioned above for the intact liver. Thus, either there is an artifactually low rate after isolation of the organelles, or the contribution by extramitochondrial sources is considerable, or there is an overestimation by the hydrogen donor titration method. Conversely, in addition to the H_2O_2 detected with the catalase Compound I method (Table 1), additional H_2O_2 flux occurs through the peroxiredoxins, thioredoxins, and GSH peroxidases (see below). These issues need to be addressed in further studies as methodology advances.

Genetically Encoded Fluorescent Protein Indicators of H_2O_2

The fluorescent probe HyPer (4) consists of circularly permuted yellow fluorescent protein (cpYFP) inserted into the regulatory domain of the prokaryotic H_2O_2-sensing protein, OxyR (hydrogen peroxide-inducible gene regulator). An illustration of the type of imaging of H_2O_2 in intact organisms is given in Fig. 2, where the time course and color intensity ascribed to H_2O_2 generation in a model of tissue injury and repair as well as proliferation are indicated (13). Several types of redox-sensitive proteins have been developed, as reviewed in Refs. 14 and 15). Major issues concern specificity and sensitivity. Nonetheless, progress in the development of these techniques has enormous potential in noninvasive investigation of physiological and pathophysiological processes. The use of H_2O_2-generating enzymes fused to HyPer is one such example; the HyPer-D-amino acid oxidase construct enables calibration and intercellular as well as subcellular analysis noninvasively (16).

"Nonredox" Exogenous Probes

Using boronate-based chemistry (17, 18), an exogenous probe compound is administered to the intact cell or organism that is then to be transformed *in vivo* to a diagnostic fluorescent compound or an "exomarker," which is analyzable by *e.g.* mass spectrometry. One such example is the use of the compound, MitoB ((3-hydroxybenzyl)triphenylphosphonium bromide), to infer levels of mitochondrial H_2O_2 (19). Peroxynitrite can also react with the boronate-based probes. Possibilities and pitfalls in using available methods to detect hydrogen peroxide in living cells were examined (20, 21).

What Are the Metabolic Sources and Sinks of H_2O_2?

Sources

A major source of hydrogen peroxide comes from the dismutation of the superoxide anion radical, formed by 1-electron reduction of oxygen. Although there is spontaneous dismutation, superoxide dismutases catalyze the reaction. Among several types of superoxide source, NAD(P)H oxidases are prominent, operating under the control of growth factors and cytokines (22). Activated monocytes or macrophages release superoxide (23), and neutrophils and eosinophils utilize oxidants in antibacterial defense (oxidative burst). Important for signaling, other cell types also exhibit controlled release of superoxide, as shown for human dermal fibroblasts treated with the proinflammatory cytokines interleukin-1 or tumor necrosis factor-α (24). Spatial and temporal analysis of NADPH oxidase-generated H_2O_2 signaling became amenable using novel fluorescence resonance energy transfer (FRET)-based reporter proteins, OxyFRET and PerFRET (25).

Another major cellular source of H_2O_2 resides in the mitochondria (26). Respiratory chain-linked H_2O_2 production (27) was attributed to superoxide radicals (28), and the mechanism of mitochondrial superoxide production by the cytochrome *bc1* complex (Complex III) has been elucidated (29). It is notewor-

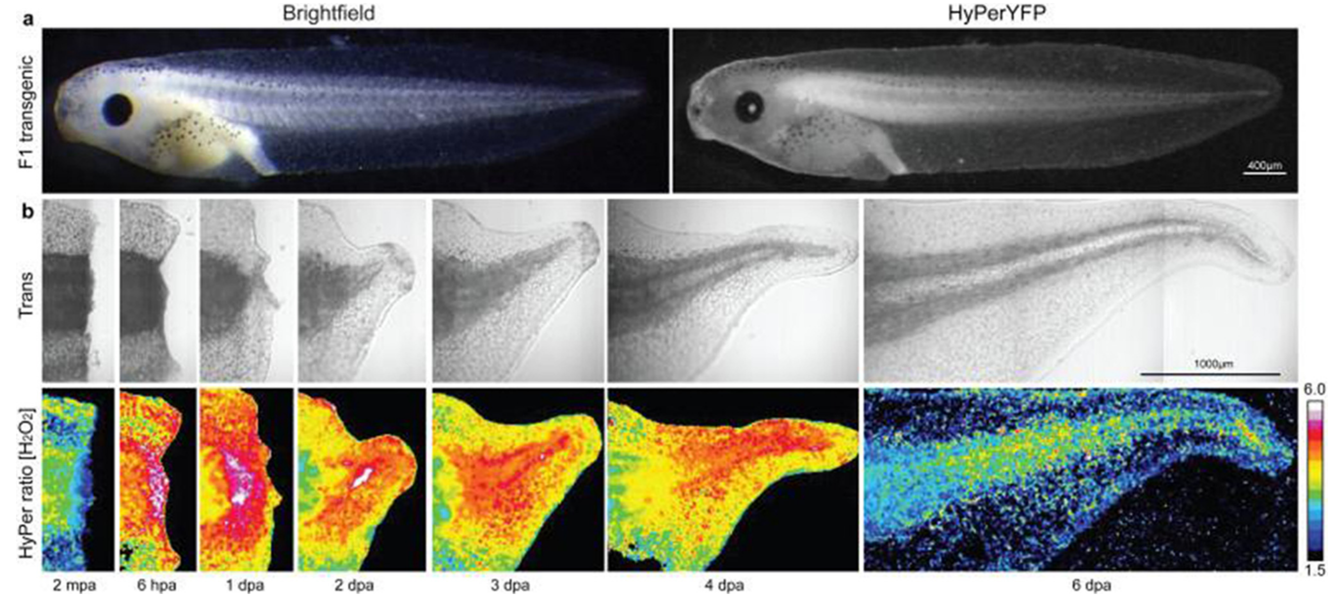

FIGURE 2. **Production of H_2O_2 during tadpole tail regeneration.** Images on the *bottom* show the false color representation of $[H_2O_2]$ at 2 min post amputation (*mpa*) of the tadpole tail and in hours (*hpa*) or days (*dpa*) post amputation. From Love *et al.* (13), with permission.

thy that Complex I is another major source of mitochondrial superoxide production and that the release of superoxide is directed toward the mitochondrial matrix space, whereas Complex III produces it toward the intermembrane space. Transitory reactivation of Complex I is a central pathological feature in ischemia-reperfusion injury. Prevention of this reactivation by modification of a cysteine switch (*S*-nitrosation of Cys-29 in the ND3 subunit) was shown to be a robust cardioprotective mechanism (30). Mitochondrial Complex II is a further independent source of mitochondrial reactive oxygen species (31). Direct production of H_2O_2 by enzymatic sources occurs by a number of oxidases, many of which operate in specific cell types and in specific subcellular compartments, such as xanthine oxidase, monoamine oxidases, or D-amino acid oxidase, to name a few (32).

Sinks

Metabolic sinks of H_2O_2 include the catalatic reaction, carried out by catalase, as well as the various peroxidatic reactions, performed as well by catalase, but importantly also by numerous peroxidases. Furthermore, in organs, the diffusion of H_2O_2 away from its source, even across membranes to the extracellular space or to other cells, is a possibility. The catalatic reaction, *i.e.* the dismutation of H_2O_2 to H_2O and O_2, may be regarded as a safety valve, occurring at higher ranges of H_2O_2 concentration, *e.g.* under toxic conditions. Catalase can also reduce H_2O_2 in the presence of metabolic hydrogen donors in the peroxidatic reaction (33). As shown in Fig. 1, external hydrogen donors such as methanol can be used to ”titrate“ catalase Compound I (8, 9). Peroxidases reduce H_2O_2 in usually highly specialized reactions. Although the flux in these peroxidase reactions may be low, their metabolic significance is considerable, in view of temporal and spatial regulation (see below).

Peroxidases of various nature are susceptible to regulation by metabolic signals. A foremost example emerged with the discovery of the peroxiredoxins (34), as reviewed (35). The 10^6-fold higher rate constant of the reaction of H_2O_2 with the cysteine thiolate (C_p) in peroxiredoxins as compared with most other deprotonated thiols (36–38) makes for a special role. Thus, under normal cellular conditions, eukaryotic peroxiredoxins were predicted to be responsible for the reduction of up to 90% of mitochondrial H_2O_2 and even more than that of cytosolic H_2O_2 (39, 40). On the other hand, cysteine residues in peroxiredoxins can become hyperoxidized to cysteine sulfinic acid, which results in an inactivation of the peroxidase. This is crucial for the sensitivity in H_2O_2 redox signaling. As a result, there is a subsequent local buildup of H_2O_2, allowing the oxidation of specific target proteins, likened to the opening of a “floodgate” (41). The functional loop is closed by sulfiredoxins, which reduce the hyperoxidized peroxiredoxins (Fig. 3) (42, 43).

Glutathione peroxidases in various subcellular compartments and cell types have a major function in the control of H_2O_2 and of other hydroperoxides (see Refs. 44 and 45). Glutathione disulfide reductase activity allows for maintenance of flux, and GSSG efflux from cells is another option. Using external H_2O_2 as challenge, the rate of GSSG efflux from liver, for example, was 3 nmol of GSSG/min/gram of wet weight at a steady-state rate of H_2O_2 infusion of 100 nmol/min/gram of wet weight (46).

H_2O_2 Compartmentation

As discussed above, the local concentration of H_2O_2 is governed by the control of its generation and of its removal. Concerning removal, the diffusion of this uncharged molecule away from the site of generation and across biomembranes leads to H_2O_2 gradients (47). High capacity of removal, *e.g.* by catalase in the peroxisomes, will generate intracellular gradients. Importantly, the local activity of peroxiredoxins near signaling sites, *e.g.* caveolae areas of the plasma membrane, will govern steady-state concentrations. Use of techniques for cell culture studies with the glucose oxidase/catalase system (48) yielded

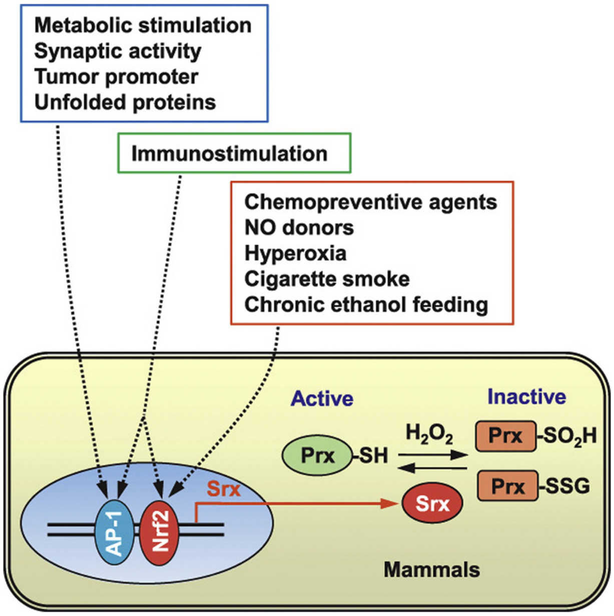

FIGURE 3. **Role of sulfiredoxin (*Srx*) as a regulator of peroxiredoxin (*Prx*) function and regulation of its expression.** Relationship to external stimuli is also shown. From Jeong *et al.* (43), with permission.

the insight that the peroxiredoxin-2 dimer-to-monomer ratio is suitable to follow the H_2O_2 steady-state concentration down to physiological levels (49).

Aquaporins as Peroxiporins

H_2O_2, a molecule with chemical and physicochemical properties close to those of H_2O, was shown to use water channels, the aquaporins, to cross the cell membrane more rapidly than by simple diffusion (50). This discovery opened an exciting field on membrane transport of hydrogen peroxide (51). Specific aquaporins facilitate the diffusion of H_2O_2 across membranes, which is why they are also referred to as peroxiporins (52). Mitochondrial aquaporin-8 knockdown in human hepatoma HepG2 cells caused loss of viability (53). Silencing of aquaporin-8 inhibited H_2O_2 entry into HeLa cells (54). Aquaporin-3 was shown to mediate H_2O_2 uptake to regulate downstream signaling (55). There are multiple interactions of aquaporins and H_2O_2 in cells, both at the intracellular-extracellular spaces, but also within subcellular compartments (56). Aquaporin-8 is able to modulate Nox (NAD(P)H oxidase)-produced H_2O_2 transport through the plasma membrane in leukemia cells (57), an interesting aspect for potential therapeutic strategies addressing H_2O_2 transport.

What Is the Role of H_2O_2 in Redox Signaling and Oxidative Stress?

Mechanism

The oxidative modification of amino acid side chains in proteins by H_2O_2 involves, in decreasing order of reactivity and biological reversibility, cysteine, methionine, proline, histidine, and tryptophan (see Ref. 58). Thiol modification is key in H_2O_2 sensing and perception in proteins (59). Transmission of a redox signal to protein thiols initiated by H_2O_2 can occur in several ways (see Ref. 37): (i) by direct oxidation of a target protein, (ii) by oxidation via a highly reactive sensor protein, (iii) by activation of a target protein upon dissociation of an oxidized inhibitor, (iv) by oxidation of a target protein via a secondary product generated through *e.g.* thioredoxin, (v) by inactivation of a scavenging protein such as peroxiredoxin to allow the oxidation of the target protein (floodgate model, see Ref. 41 above), and (vi) by association of the target protein with an H_2O_2-generating protein to allow site-directed oxidation. In addition to direct oxidation, protein glutathionylation and other modifications can occur and serve in redox signaling.

Targets

Insulin signaling was probably the first transduction chain in which H_2O_2 was invoked as a second messenger (60); H_2O_2 was called an "insulinomimetic" (61). Growth factors such as platelet-derived growth factor (PDGF) (62), through H_2O_2 production, induce downstream effects on tyrosine phosphorylation, as do other important growth factors such as epidermal growth factor (EGF) (63), fibroblast growth factor (FGF) (64), or vascular endothelial growth factor (VEGF) (65). A major mechanism is the inactivation of protein phosphatases by H_2O_2, thereby increasing the level of protein phosphorylation. Also, direct modification of the EGF receptor by H_2O_2 at a critical active site cysteine (Cys-797) was shown to enhance tyrosine kinase activity (66).

Regarding nonreceptor kinases, signal-mediated H_2O_2 production increases Akt (also known as protein kinase B (PKB)) activation (67). Another group of serine/threonine kinases, the MAP kinases, mediate redox modulation of Erk1/2, JNK, and p38. As comprehensively reviewed in Ref. 68, many studies documented H_2O_2-induced activation of MAPK pathways, and the redox-based inactivation of upstream components also serves to modulate MAPK signal duration. Critical thiols are centrally involved in activation of essential switches in defense reactions, namely in the NF-κB (69) and Nrf2/Keap1 (70) systems, important in chemoprevention and cytoprotection (71). The nature of targets extends from the specific ones mentioned above to reactive cysteines in general, a wide open field of research on sulfur switches, governing the set point in the protein-cysteine redox proteome (72–74).

Processes

The functional consequences of H_2O_2 signaling concern fundamental biological processes. The role of mitochondrial H_2O_2 was recently discussed (75) for hypoxia, inflammation, apoptosis, and autophagy. Concepts of the inflammasome (76) and redoxosome (77) have evolved. In wound healing, H_2O_2 signaling has been established as a prominent early feature (1, 78, 79), shown for the wound healing/proliferation model in Fig. 2. H_2O_2 acts as a chemoattractant (78, 80). New horizons have been opened in understanding the intricate relationships of reactive oxygen species in immunology (81).

Much has to be learned for better understanding the role of redox signaling in metabolism, in insulin signaling in particular (82). Although reactive oxygen species enhance insulin signaling (83), excessive levels may cause diabetic complications, so that these opposing actions constitute a "peroxide dilemma" (84, 85).

The current perception of the aging process includes a role of metabolic alterations such as dysregulated nutrient sensing and mitochondrial dysfunction, all of which encompass alterations in H_2O_2 signaling. Intracellular H_2O_2 concentration in skeletal muscle rises by about 100 nM during contractions (86). This response is weakened in aging, which may contribute to age-related loss of muscle mass and to frailty (86). An interesting aspect of redox regulation in aging is the cellular polarity, mediated by the activity of AMP-activated protein kinase (AMPK) in controlling the cytoskeleton (87). Peroxiredoxins are conserved markers of circadian rhythm (88), and chronobiological research has revealed a tight coupling of redox reactions to circadian rhythmicity (89).

Oxidative Stress

The initial concept of oxidative stress focused on the damage of biomolecules such as DNA, lipids, and proteins (58). It was extended to include the emerging role of biologically generated oxidants in redox signaling (90): "Oxidative stress is an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage." With the recognition of the role of low level oxidant stimuli for altering the set point of gene expression for batteries of enzymes, known as hormesis (91), physiological oxidative stress came into focus on a spatial and temporal dimension. Tissue-scale gradients and regional specificity are being identified (78, 92).

Concluding Remarks

Retrospective

The occurrence of H_2O_2 in normal aerobic metabolism was heavily contested in the early days of research in bioenergetics, with the quote from the 1920s in the Warburg-Wieland dispute "that even after killing a whole dog there was not one drop of H_2O_2 detectable." In addition, Keilin and Hartree in 1945 (33) stated: "Contrary to the view that H_2O_2 is generally formed in cells and tissues during respiratory processes are the following two facts . . . " and Britton Chance in 1951 (93) concluded: "Quantitative evidence for the existence of significant amounts of . . . H_2O_2 . . . in tissue is lacking, since catalase, by virtue of its peculiar capacity for catalatic reactions literally 'destroys the evidence' of free hydrogen peroxide in the cell." It was not until the continuous detection of catalase Compound I in intact tissue under steady-state conditions that H_2O_2 production was proven in 1970 (6). It might be appropriate to quote the final sentence in the review on hydroperoxide metabolism in mammalian organs from 1979 (10): "Finally, recent understanding of the beneficial action of H_2O_2 in phagocytosis and in ethanol oxidation suggests caution in condemning any metabolite as useless until its functions *in toto* are thoroughly understood."

Prospective

The advent of novel converging techniques from cell biology, noninvasive imaging for H_2O_2 detection, and metabolic studies opened a new vista. Hopefully, there will be real-time spatially resolved quantitative monitoring of H_2O_2 as a versatile and innocuous oxygen metabolite functioning in redox signaling. Appropriate control is provided by the powerful generators, scavengers, and switches discussed above. H_2O_2 serves as a central hub for information flow in plant cells as well (94), and there is indication that waves of H_2O_2 transmit information in plant cells (95). At present, it still appears puzzling how local fine-tuning is orchestrated in the simultaneous presence of a multitude of potential reactants. Shaping the microenvironment for the recruitment of target proteins to the site of H_2O_2 production, and *vice versa*, is one of the strategies. A concept has been proposed (96) of ”redox optimization“ between mitochondrial respiration and formation of reactive oxygen species. More refinement of methodology for noninvasive detection of H_2O_2 production by cellular NADPH oxidases is required (97). The threshold from signaling to excessive toxic levels will be challenging to further identify. The precise transition points for these cellular responses may vary due to cell type and metabolic conditions (see Ref. 2).

Note: This minireview focused on aspects of metabolic H_2O_2 generation. Xenobiotic and toxicological sources such as in ”redox cycling“ and lipid peroxidation (98) were not considered here. Further, it should be mentioned that redox signaling extends to other large and important sectors, only one example being that of peroxynitrite biology and the field of protein tyrosine nitration (99, 100). It will be another challenging area of research to analyze the cross-talk and interrelationships between different modalities of redox signaling.

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