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Review

Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide ☆

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ABSTRACT

Background: Hydrogen peroxide (H_2O_2) is an important signaling compound that has recently been identified as a new substrate for several members of the aquaporin superfamily in various organisms. Evidence is emerging about the physiological significance of aquaporin-facilitated H_2O_2 diffusion.
Scope of review: This review summarizes current knowledge about aquaporin-facilitated H_2O_2 diffusion across cellular membranes. It focuses on physicochemical and experimental evidence demonstrating the involvement of aquaporins in the transport of this redox signaling compound and discusses the regulation and structural prerequisites of these channels to transmit this signal. It also provides perspectives about the potential importance of aquaporin-facilitated H_2O_2 diffusion processes and places this knowledge in the context of the current understanding of transmembrane redox signaling processes.
Major conclusions: Specific aquaporin isoforms facilitate the passive diffusion of H_2O_2 across biological membranes and control H_2O_2 membrane permeability and signaling in living organisms.
General significance: Redox signaling is a very important process regulating the physiology of cells and organisms in a similar way to the well-characterized hormonal and calcium signaling pathways. Efficient transmembrane diffusion of H_2O_2, a key molecule in the redox signaling network, requires aquaporins and makes these channels important players in this signaling process. Channel-mediated membrane transport allows the fine adjustment of H_2O_2 levels in the cytoplasm, intracellular organelles, the apoplast, and the extracellular space, which are essential for it to function as a signal molecule. This article is part of a Special Issue entitled Aquaporins.

1. Introduction

Major intrinsic proteins (MIPs) form a large family of transmembrane channel proteins, and *MIP* genes have been found in the genomes of vertebrates, insects, plants, fungi, protozoa, bacteria, and viruses, thus covering all kingdoms of life [1]. The first functional characterization of a MIP protein, CHIP28, later renamed aquaporin 1 (AQP1), from human red blood cells, was performed by Preston in Agre's laboratory [2], who showed that it was a functional water channel when heterologously expressed in *Xenopus laevis* oocytes and that AQP1 was the hydrophilic pore hypothesized by Stein and Danielli in 1956 [3] to explain the high membrane water permeability of red blood cells and other cells, which could not be explained solely by simple passive diffusion of water molecules across the lipid bilayers. The crucial role of MIPs in water transport processes became clear, and the name "aquaporin" (AQP) was rapidly adopted for MIPs transporting water and, subsequently, for the whole MIP family.

Based on sequence homologies, MIPs are divided into two clades, the mainly water-permeable AQPs, forming the AQP clade, and the mainly glycerol-permeable aquaglyceroporins, forming the glycerol facilitator-like protein (GLP) clade [1]. Structural, biochemical, and molecular studies demonstrated that, despite their sequence diversity, all MIPs have a molecular mass of 26–32 kDa and consist of cytoplasmic N and C termini and six membrane spanning helices connected by five loops (A to E). Loops B and E contain the highly conserved MIP asparagine–proline–alanine signature sequence and these motifs meet in the middle of the membrane, forming a narrow hydrophilic path [4]. A second selectivity filter, the so-called aromatic/arginine (ar/R) constriction region, formed by four amino acids, functions as a size exclusion barrier and forms the hydrogen bond environment necessary for the efficient transport of the substrate [4]. Each protein forms a hydrophilic bidirectional channel pathway through which substrates move according to their chemical gradient across the membrane. MIPs form tetramers, in which each monomer is a functional channel.

In the years following the discovery of water- and glycerol-permeable AQPs, a variety of metabolically important small uncharged solutes were identified as AQP substrates, i.e. urea [5,6], carbon dioxide

Abbreviations: AQP, aquaporin; ASMC, aortic smooth muscle cells; CM-H_2DCFDA, (5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester; EGF, epidermal growth factor; GLP, glycerol facilitator-like protein; HDCFDA, 2′7′-dichlorodihydrofluorescein diacetate; HEK 293, human embryonic kidney 293; H_2O_2, hydrogen peroxide; MIP, major intrinsic protein; NIP, nodulin26-like intrinsic protein; PIP, plasma membrane intrinsic protein; PY1-ME, peroxy yellow 1 methyl ester; ROS, reactive oxygen species; TIP, tonoplast intrinsic protein; XIP, X intrinsic protein

☆ This article is part of a Special Issue entitled Aquaporins.

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Table 1
Major intrinsic proteins facilitating the membrane diffusion of hydrogen peroxide.

Aquaporin	Organism	Phylogenetic clade	Functional assay system	Protein localization	Putative function	Reference
AtPIP2;1	Plant	Aquaporin	Toxicity growth assay using yeast Uptake assay using yeast Molecular simulation study	Plasma membrane	H_2O_2 signaling	[14]
AtPIP2;2	Plant	Aquaporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 signaling	[71]
AtPIP2;4	Plant	Aquaporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 signaling	[14] [71]
AtPIP2;5	Plant	Aquaporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 signaling	[71]
AtPIP2;7	Plant	Aquaporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 signaling	[71]
ZmPIP2;5	Plant	Aquaporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 signaling	[68]
AtTIP1;1	Plant	Aquaporin	Toxicity growth assay using yeast Uptake assay using yeast	Tonoplast	H_2O_2 detoxification	[13]
AtTIP1;2	Plant	Aquaporin	Toxicity growth assay using yeast Uptake assay using yeast	Tonoplast	H_2O_2 detoxification	[13]
TgTIP1;1	Plant	Aquaporin	Toxicity growth assay using yeast	Tonoplast	H_2O_2 detoxification	[69]
TgTIP1;2	Plant	Aquaporin	Toxicity growth assay using yeast	Tonoplast	H_2O_2 detoxification	[69]
AtTIP2;3	Plant	Aquaporin	Toxicity growth assay using yeast	Tonoplast	H_2O_2 detoxification	[14]
AtNIP1;2	Plant	Aquaporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 signaling	[14]
NtXIP1;1α	Plant	Aquaporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 signaling	[70]
NtXIP1;1β	Plant	Aquaporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 signaling	[70]
StXIP1;1α	Plant	Aquaporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 signaling	[70]
StXIP1;1β	Plant	Aquaporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 signaling	[70]
SlXIP1;1α	Plant	Aquaporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 signaling	[70]
SlXIP1;1β	Plant	Aquaporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 signaling	[70]
HsAQP8	Mammal	Aquaporin	Toxicity growth assay using yeast Uptake assay using yeast Uptake assay using HEK 293 cells Uptake assay using HEK 293 cells Transport assay using isolated mitochondria from human hepatoma HepG2 cells	Plasma membrane Inner mitochondrial membrane	H_2O_2 signaling H_2O_2 detoxification	[13] [88] [65] [72]
HsAQP3	Mammal	Aquaglyceroporin	Uptake assay using HEK 293 cells Uptake assay using HeLa cells Uptake assay using Ht29 cells Uptake assays using $CD4^+$ T cells from $AQP3^{+/+}$ and $AQP3^{-/-}$ mice Signaling assay using human T lymphocytes	Plasma membrane	H_2O_2 signaling	[80] [82] [82]
RnAQP1	Mammal	Aquaporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 signaling	E. Beitz, personal communication [87]
LpGlpF1	Bacterium	Aquaglyceroporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 detoxification	[12]
LpGlpF3	Bacterium	Aquaglyceroporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 detoxification	[12]
LpGlpF4	Bacterium	Aquaglyceroporin	Toxicity growth assay using yeast	Plasma membrane	H_2O_2 detoxification	[12]

At, *Arabidopsis thaliana*; Hs, *Homo sapiens*; Lp, *Lactobacillus plantarum*; Nt, *Nicotiana tabacum*; Rn, *Rattus norvegicus*; Sl, *Solanum lycopersicum*; St, *Solanum tuberosum*; Tg, *Tulipa gesneriana*; Zm, *Zea mays*.

[7,8], nitric oxide [9], lactic acid [10–12], hydrogen peroxide (H_2O_2) (Table 1) [13,14], acetic acid [15], ammonia [16,17], arsenite [18–21], boric acid [22], silicic acid [19], antimonite [18,21], and selenite [23]. It was also demonstrated that AQPs play physiologically important roles in the uptake, translocation, sequestration, or extrusion of these molecules (reviewed in [24]).

In this review, we describe the discovery of H_2O_2-channeling AQPs, summarize the physicochemical and experimental evidence for their involvement in H_2O_2 transmembrane transport, discuss the known regulatory interplay of these channels and H_2O_2, and provide a perspective for their roles in the context of our current understanding of transmembrane redox signaling processes.

2. Hydrogen peroxide is an important signaling molecule

During its life span, each organism is confronted with a vast set of temporal, spatial, and environmental cues that affect its development and physiology and require appropriate and coordinated responses. Organisms have developed a repertoire of independent and/or linked signaling pathways in order to control all normal developmental events and to react adequately to all unexpected challenges. Local and systemic signaling pathways involve calcium ions and reactive forms of the oxygen molecule, collectively referred to as reactive oxygen species (ROS). ROS activate components of diverse signaling pathways, such as hormones, kinases/phosphatases, and metabolites, which, in turn, trigger the expression of specific genes required for cellular and metabolic adaptation to new environmental conditions or a new developmental stage. The production of ROS in different subcellular compartments (apoplast, chloroplast, mitochondria, and endoplasmic reticulum) is a characteristic of the signaling responses to biotic and abiotic stress stimuli and to developmental cues and triggers both apoptosis and cell proliferation (reviewed in [25–28]). ROS are not only considered as cell damaging and destructive compounds, but also found to be crucial signaling molecules regulating various important responses and metabolic pathways [26]. This is also demonstrated by the observation that ROS levels inducing detrimental cell damage or death are often far lower than those considered to be toxic [29]. Only one or a few types of ROS-activated proteins may be sufficient to switch on a genetic program resulting in cell death.

ROS, including singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet-}$), H_2O_2, and the hydroxyl radical ($^{\bullet}OH$), are unavoidable by-products of aerobic metabolism in mitochondria and chloroplasts and of other cellular redox reactions [30–32]. H_2O_2 is one of the most abundant and stable ROS molecules in organisms. Due to its intermediate oxidation number (-1), it possesses reducing and oxidizing properties that are important for its cellular multi-functionality. H_2O_2 can be produced from, and converted to, other ROS. H_2O_2 can directly or indirectly (via the formation of other ROS) contribute to DNA or lipid peroxidation, protein

carbonylation, and the oxidation of methionine residues and thiol groups of cysteines to sulfenic, sulfinic, or sulfonic derivatives; the last two groups of modifications result in a change in the redox status of the proteins and either activate or inactivate the protein or alter its function [33].

H_2O_2 acts as a signaling molecule in various cellular processes as either a paracrine (intercellular) or an autocrine (intracellular) signal. An intercellular signaling function means that the signal molecule needs to be transported across at least one membrane. The extracellular enzyme activities of cell wall peroxidases [34] and plasma membrane NADPH oxidases [35,36] result in the generation of extracellular ROS, including H_2O_2, which regulate a large set of developmental and physiological processes and stress responses within cells. In both plant and mammalian cells, H_2O_2 can either directly trigger chemical reactions and influence responsive targets, such as metabolites or proteins, or act via signaling pathways often involving MAPK kinases (reviewed in [37–40]). H_2O_2, which is produced in membrane-surrounded organelles, such as mitochondria, chloroplasts, peroxisomes, nuclei and the endoplasmic reticulum, has been demonstrated to act as a signal molecule outside these organelles [41–48].

In both the inter- and intracellular signaling processes, regulatory mechanisms facilitating the membrane diffusion of the signaling molecule are required. In many cases, experimental findings have suggested, or demonstrated, that AQPs are responsible for the facilitated diffusion of H_2O_2 across membranes (Table 1).

3. Transport of hydrogen peroxide

3.1. The diffusion of hydrogen peroxide across membranes is limited

For a long time, it was thought that H_2O_2 freely crosses membranes only by passive non-protein-facilitated diffusion. However, H_2O_2 gradients have been reported across the membranes of mammalian cell lines [49,50], bacteria [51], and yeasts [52,53], in agreement with the physicochemical properties of H_2O_2, which has a slightly larger dipole moment/polarity than water, making its non-facilitated diffusion through the hydrophobic lipid bilayer less rapid than that of water. In the case of water, AQPs facilitate its transmembrane diffusion to meet the physiological demands of sufficiently fast fluxes, which would be impossible to achieve by non-facilitated free diffusion across biological membranes. As the chemical properties of H_2O_2 and experimental studies suggest that biological membranes generally prevent the free diffusion of H_2O_2 even more than that of water, active regulation is needed to adjust membrane permeability to H_2O_2.

3.2. The chemical composition of membranes influences the transmembrane diffusion of hydrogen peroxide

The permeability of biological membranes to polar and lipophilic compounds depends on their biophysical properties and chemical composition, including the length and saturation of the fatty acids and the amount, composition, and degree of phosphorylation and glycosylation of proteins [54–56]. For instance, permeability of the plasma membrane to H_2O_2 was shown to be higher in exponentially growing wild-type yeast than in cells in stationary phase, and to be altered in yeast with mutated ergosterol biosynthesis genes (*erg3Δ*, *erg6Δ*) compared to wild-type cells, demonstrating that H_2O_2 permeability is dependent on the sterol composition of the plasma membrane and possibly on the reorganization of ergosterol microdomains during growth [52,57]. Later, it was shown that addition of H_2O_2 to wild-type yeast, but not *Δerg3* or *Δerg6* mutants, induced a rapid increase in the anisotropy of the plasma membrane, followed by changes in the H_2O_2 permeability [58], demonstrating that H_2O_2 permeability is affected by modulation of the biophysical properties of the plasma membrane. Pedroso et al. [57] confirmed that the plasma membrane plays an essential role in yeast adaptation to H_2O_2 and showed, using a proteomic approach, that proteins involved in lipid and vesicle trafficking are important mediators of H_2O_2 adaptation.

3.3. AQPs control the transmembrane diffusion of hydrogen peroxide

3.3.1. Hydrogen peroxide and water share physicochemical features that are important for their passage through an AQP channel

Whether a substrate can move through an AQP is determined by the physicochemical environment generated by the amino acids facing the channel path. The size and volume of the substrate are obviously important factors for transport ability and capacity. If a substrate is too large, it cannot traverse the channel. On the other hand, the flux of molecules smaller than the diameter of the channel is also restricted if no energetically favorable intermolecular interactions occur between the channeled substrate and the carbonyl backbone of the amino acids lining the pore. In this respect, the ability of the substrate to form energetically favorable hydrogen bonds with certain residues is essential for its passage through an AQP [59]. Obviously, substrates that form too strong interactions with the protein will also not permeate through the channel. Thus, the electrochemical properties of the substrate, such as its dipole moment and surface charge distribution, are of great importance for efficient passage. All of these features make H_2O_2 a possible AQP substrate (reviewed in [60]), as it has almost the same dipole moment (2.26×10^{-18} esu [H_2O_2] vs. 1.85×10^{-18} esu [H_2O]), dielectric constant (73.1 [H_2O_2] vs. 80.4 [H_2O]), molecular diameter (0.25–0.28 nm [H_2O_2] vs. ca. 0.275 nm [H_2O]), and ability to form hydrogen bonds as water (reviewed in [60]).

The thermodynamically unexpected permanent dipole moment of H_2O_2, measured by the Stark effect in the microwave spectrum, is even higher than that of water, suggesting a skewed cis conformation of H_2O_2, in which both oxygen–hydrogen bonds are at 97° to the oxygen–oxygen bond, the two hydrogen atoms being in two different planes oriented at an angle of 94° to each other and the lone electron pairs oriented in the opposite direction to the hydrogen atoms (Fig. 1) [61]. Depending on the physical state of H_2O_2, these angles vary, but always result in a non-planar orientation of the molecule. With its two pKa values of about 11 and 16 [62], H_2O_2 is uncharged and protonated at physiological pH ranges, which is one prerequisite for it to be transported by AQPs. These significant structural similarities of H_2O_2

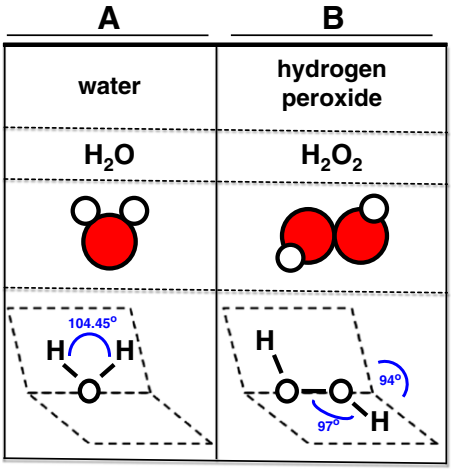

Fig. 1. Water and hydrogen peroxide. Chemical formula, a drawing of the molecular structure, and the steric arrangement of water (A) and hydrogen peroxide (B) (according to [61]). Oxygen and hydrogen atoms are shown, respectively, as red or white circles. The angles in blue illustrate the skewed cis conformation of H_2O_2, in which both oxygen–hydrogen bonds are at 97° to the oxygen–oxygen bond, the two hydrogen atoms being in two different planes oriented at an angle of 94° to each other and the lone electron pairs oriented in the opposite direction to the hydrogen atoms, which results in the polarity of H_2O_2.

and water represent the physicochemical basis for AQP-mediated H_2O_2 transport.

3.3.2. Indirect experimental evidence for AQP-mediated facilitated transmembrane diffusion of H_2O_2

Long before it was experimentally demonstrated that specific AQPs facilitate H_2O_2 diffusion, this transport ability was hypothesized, mainly based on experiments in which AQP inhibitors, such as mercuric chloride ($HgCl_2$), silver nitrate, or phloretin, were found to significantly inhibit the transmembrane flux of H_2O_2 [13,63–65]. One groundbreaking study analyzed the transmembrane diffusion of water and H_2O_2 in parallel in internodal cells of the freshwater algae *Chara corallina* [63]. The permeability coefficient of H_2O_2, which is a measure of the speed with which a compound crosses a given membrane, was found to be very close to that of water, and treatment with $HgCl_2$ significantly decreased the influx of both molecules into the cells. In addition, the relatively high reflection coefficient for H_2O_2 was explained by the assumption that some, but not all, AQPs function as H_2O_2 facilitators, and these were named "peroxoporins" by Henzler and Steudle [63].

An effect of AQP inhibitors is still commonly used to suggest that AQPs determine the transmembrane diffusion of H_2O_2 [65,66]. A fraction of the H_2O_2 produced in chloroplasts diffuses into the cytoplasm, where it either activates signaling pathways or is degraded [67]. However, the mechanism of H_2O_2 diffusion through the chloroplast envelope is still a matter of debate. Resorufin fluorescence resulting from the reaction of Amplex Red with H_2O_2 catalyzed by a peroxidase outside the chloroplast is decreased by 60% in the presence of acetazolamide, an AQP inhibitor [64], suggesting that AQPs, such as NtAQP1 from *Nicotiana tabacum* [7], in chloroplast membranes might be the pathway by which H_2O_2 exits the chloroplast.

3.3.3. Direct experimental evidence for AQP-mediated facilitated transmembrane diffusion of H_2O_2

In 2007, to directly determine whether AQPs had the ability to facilitate the diffusion of H_2O_2 across cellular membranes, AQPs and aquaglyceroporins from mammals and AQPs from four subfamilies found in higher plants [plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), small basic intrinsic proteins, and nodulin26-like intrinsic proteins (NIPs)] were screened [13]. Exposure of different yeast strains that differed in their sensitivity to oxidative stress to increasing concentrations of H_2O_2 resulted in various degrees of decreased growth and cell survival depending on the ability of the strain to detoxify ROS, and the heterologous expression of most AQP isoforms, including hAQP1, a water-specific AQP, in these strains did not significantly change their sensitivity to increasing concentrations of H_2O_2. However, expression of human hAQP8, *Arabidopsis* AtTIP1;1 and AtTIP1;2 markedly reduced growth and cell survival on medium containing H_2O_2. Similar findings in several independent yeast (wild-type and mutant) strains suggested that the decreased tolerance to H_2O_2 in the medium was not a secondary effect on a given yeast strain. To exclude the possibility that the increased sensitivity of hAQP8- or AtTIP1-expressing yeasts was not due to a decreased ROS detoxification ability, the authors showed that AQP expression did not lead to a reduction in intrinsic key H_2O_2-detoxifying enzymes [13]. Later on, when investigating the reason for the ability of ZmPIP2;5, in contrast to ZmPIP1;2, to channel H_2O_2 in yeast cells, despite having identical selectivity filters, we demonstrated the need to design appropriate control experiments to show that the protein is expressed (western blot) and localized correctly before concluding on transport activity [68].

However, growth inhibition following supplementation of the growth medium with H_2O_2 is a rather slow and indirect approach. To more directly follow H_2O_2 transport across the yeast plasma membrane, a fluorescence-based method for monitoring cytoplasmic H_2O_2 levels was developed using intact yeast cells and an intracellular ROS-sensitive fluorescent dye, 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) [13]. In the absence of H_2O_2, dye-loaded yeast cells transformed with *AtTIP1;1*, *hAQP1*, *hAQP8*, or empty vector did not show any significant difference in fluorescence and addition of 0.1 mM H_2O_2 to control or hAQP1-transformed yeast cells only marginally increased the fluorescence, probably as a result of passive influx of H_2O_2 across the lipid bilayer. In contrast, addition of H_2O_2 to yeast cells expressing AtTIP1;1, AtTIP1;2, or hAQP8 significantly increased the intracellular fluorescence [13]. Further evidence for AQP-mediated H_2O_2 diffusion was obtained using the fluorescence method on single yeast cells and following the uptake of H_2O_2 by confocal microscopy. Yeast cells transformed with either empty vector or vector containing *hAQP1*, *hAQP8*, or *AtTIP1;1* were preloaded with CM-H_2DCFDA and a signal was clearly detected in the cytoplasm and the region around the nucleus. On addition of H_2O_2, the fluorescence intensity increased several fold in cells transformed with *hAQP8* or *AtTIP1;1*, but not in those transformed with *hAQP1* or empty vector [13]. These experiments provided the first clear molecular genetic evidence for H_2O_2 transport through specific members of the AQP family.

Dynowski et al. [14] used a similar combination of experimental approaches to determine whether plant AQPs transport H_2O_2. The authors performed a toxicity growth assay using wild-type yeast and the mutant strain Δ*yca1*, which shows impaired H_2O_2-induced apoptosis and, thus, has lower endogenous H_2O_2 levels, and used the redox-sensitive dye 2′7′-dichlorodihydrofluorescein diacetate (HDCFDA) to monitor H_2O_2 uptake into yeast cells in suspension. Isoforms belonging to different plant AQP subfamilies (AtTIP2;3, AtNIP1;2, AtPIP2;1, and AtPIP2;4) were shown to transport H_2O_2. The authors then focused on AtPIP2;1 and, using a mutagenesis approach, showed that mutation of residues important for the water selectivity and gating of AQPs abolished the H_2O_2 sensitivity of AtPIP2;1-expressing yeast. They also analyzed the conduction of water and H_2O_2 using molecular dynamics simulations based on the crystal structure of SoPIP2;1 from spinach and showed that the energy barriers for H_2O_2 along the channel path were, at most points, a little higher than those for water, but the maximal barriers for both were seen at a similar location [14]. The dipole moment of water or H_2O_2 forces the molecules into a preferential orientation within the channel, especially at the sites of the maximal energy barriers. The residency time for H_2O_2 was maximal in the ar/R selectivity filter which is crucial for substrate selectivity, indicating a major energy barrier for H_2O_2 conduction. Mutations that increased the pore diameter in this region showed increased H_2O_2 conduction/sensitivity [14]. Taken together, these modeling and experimental data demonstrate that PIP2s determine the efficiency of H_2O_2 diffusion between cells and are in agreement with the results of Bienert and coworkers [13] showing that AQPs represent important transmembrane pathways for H_2O_2 in organisms (Fig. 2).

The combined use of the growth and survival assay for AQP-expressing yeast cells and the H_2O_2-detecting fluorescence assay established by Bienert et al. [13] and Dynowski et al. [14] has become a standard method for qualitatively assessing AQP-mediated H_2O_2 diffusion [68–71]. Several bacterial MIP isoforms and members of the plant XIP subfamily were also postulated to channel H_2O_2 because of their ability to induce increased H_2O_2 sensitivity when expressed in yeast cells [68,70]. The discovery that hAQP8 and TIP1s were particularly permeable to H_2O_2 was confirmed in several independent studies [65,69,70,72]. Interestingly, a recent phylogenetic analysis of the MIP superfamily revealed that AQP8 and TIP1s share high sequence similarity, which might be explained by a common ancestral progenitor [73,74]. The common substrate spectrum (water and H_2O_2) of these isoforms from different organisms further suggests a correlation between phylogenetic relationships and functionality.

3.3.4. Are water-permeable AQPs necessarily permeable to hydrogen peroxide?

The physicochemical similarities between water and H_2O_2 suggest that certain AQPs show the same capacity and selectivity for transporting

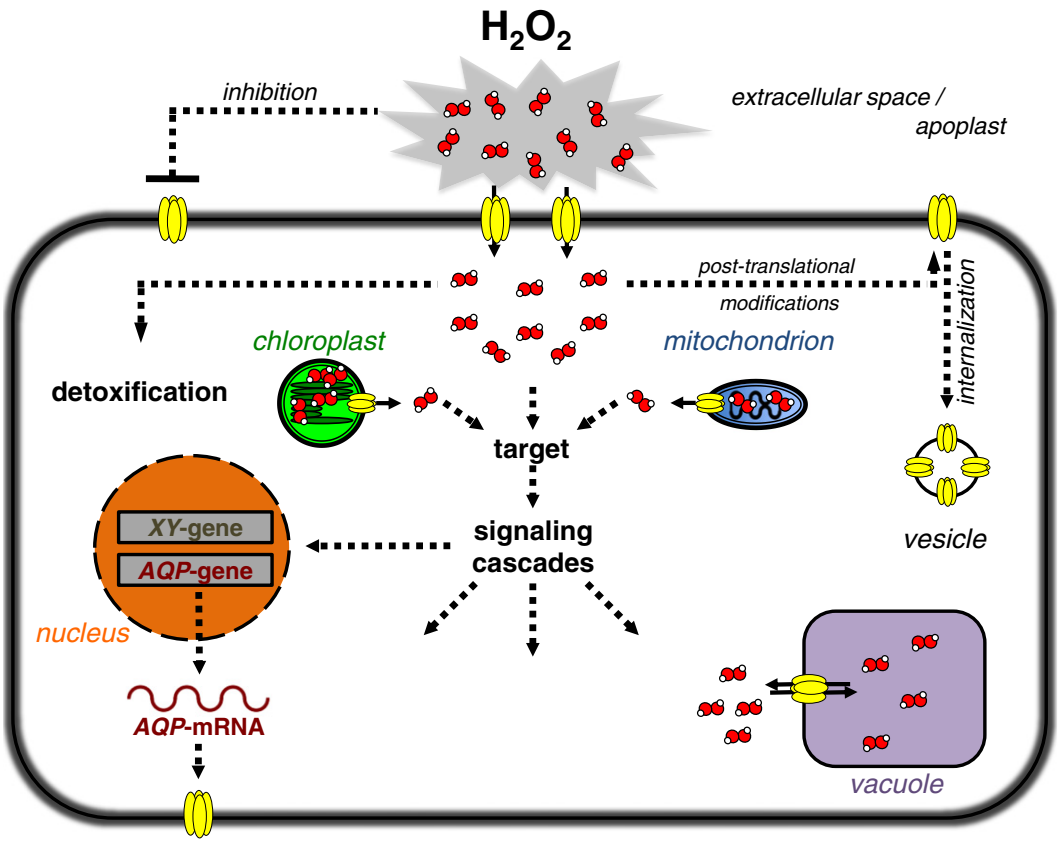

Fig. 2. Interaction of AQPs and hydrogen peroxide in cells. Extracellularly produced H_2O_2 (red) is taken up into the cell via AQPs (yellow). Inside the cell, it acts on various protein and non-protein targets, which themselves regulate diverse signaling cascades. Expression of certain AQP isoforms is suggested to be activated by H_2O_2-induced signaling cascades. H_2O_2, which is constantly produced in chloroplasts and mitochondria, is suggested to exit these organelles via AQPs. Vacuolar AQPs have been demonstrated to be highly permeable to H_2O_2 and are speculated to function in detoxification processes. H_2O_2 has been shown to inhibit AQP-mediated transmembrane transport of water, either by a direct inhibitory effect on the channel protein or by indirect regulation mechanisms (e.g. internalization and posttranslational modification). The solid black arrows indicate AQP-mediated transmembrane transport of H_2O_2 and the dashed black arrows indicate a non-specified signaling event.

these two substrates [60]. Furthermore, water and H_2O_2 appear to use the same pathway through the channel and their transport is inhibited by the same mutations, suggesting that water-permeable AQPs also facilitate H_2O_2 diffusion [14]. Indeed, hAQP8 and AtTIP1;1, which are highly permeable to H_2O_2 [13], are also very efficient water channels [75,76]. AQPs were demonstrated to allow the passage $>10^9$ water molecules per channel and per second [4,77–79]. However, current data does not allow the conclusion to be drawn that the water transport capacity of AQPs is quantitatively equivalent to their H_2O_2 permeability. For example, expression of *Solanaceae* XIPs in yeast induced a high sensitivity to externally supplied H_2O_2, even though these XIPs have no significant water transport ability [70]. On the other hand, since all PIP2s are good water channels and do not differ in the amino acid residue composition of their selectivity filters, we might wonder why AtPIP2;2, AtPIP2;4, AtPIP2;5, and AtPIP2;7 facilitate H_2O_2 membrane diffusion, while AtPIP2;3, AtPIP2;6, and AtPIP2;8 do not [71]. Whether the observed quantitative differences in H_2O_2 permeability between various AQP isoforms are due to differences in the open probability of the channels, to (mis)targeting to cellular membranes other than the plasma membrane, or to protein stability remains often experimentally unclear. At the channel level, molecular dynamics simulation showed that, at least for plant SoPIP2;1, water is faster channeled than H_2O_2 [14]. Indeed, the entrance of H_2O_2, as well as the conductance, was slightly less favorable compared with water. Only 15% of H_2O_2 molecules crossed the channel after 4 ns compared to 64% for water molecules, but all of them finally crossed it after 16 ns simulations [14]. It will be interesting to determine the selectivity determinants for water vs. H_2O_2 or any other co-channeled AQP substrate for which the physiological cellular concentrations are usually far below that of water. When investigating why PIP2s are permeable to H_2O_2, while PIP1s are not, despite having identical selectivity filters, we demonstrated the need to design appropriate control experiments showing that the protein is correctly expressed and localized before drawing any conclusions on transport activity [68]. However, the lack of significant H_2O_2 permeability of hAQP1 observed in the toxicity growth assay and in direct uptake studies cannot be questioned, as hAQP1 was shown to be expressed in the yeast plasma membrane and to be highly permeable to water therein [13]. E. Beitz and co-workers (personal communication) addressed the intriguing water vs. H_2O_2 selectivity of hAQP1 in more detail and showed that, in contrast to the expression of hAQP1, expression of rat rAQP1 in yeast did significantly increase cell sensitivity to externally supplied H_2O_2, although the two AQP1s differ in only 18 amino acids. Expression of rAQP1 also resulted in a higher water permeability of the yeast vesicles than hAQP1 expression. Lower levels of hAQP1 than rAQP1 might partly explain the different water and H_2O_2 transport efficiencies, but does not explain why only a minor transport difference is observed for water, whereas a major difference is seen for H_2O_2 transport. A different AQP1 threshold level for the detection of either water or H_2O_2 transport capacity might explain these observations. However, to date, no such threshold has been clearly identified in the yeast plasma membrane for any transport phenotype. Future studies addressing this aspect will be of great interest. To better understand the structural and (bio)chemical selectivity mechanisms leading to water and/or H_2O_2 permeability, the swapping of corresponding regions from different proteins and mutagenesis experiments using rAQP1 and hAPQ1 would be very interesting.

3.3.5. Physiological evidence for AQP-mediated facilitated transmembrane diffusion of hydrogen peroxide in mammalian cells

3.3.5.1. Role of AQP3 in the transmembrane diffusion of hydrogen peroxide. Although it had been shown that AQP blockers inhibit the flux of H_2O_2 in native membranes and that specific AQP isoforms can facilitate H_2O_2 membrane diffusion when expressed in heterologous expression systems, it remained to be demonstrated that this process occurred in homologous cell systems. In 2010, Miller and co-workers [80] provided this evidence using mammalian cells. Human embryonic kidney 293 (HEK 293) cells, a widely used model cell system, were transfected with either a control vector or vectors leading to the overexpression of hAQP1, hAQP3, or hAQP8. Only hAQP8 had previously been shown to be permeable to H_2O_2 when expressed in the yeast system [13]. The AQP-expressing and control cells were loaded with peroxy yellow 1 methyl-ester (PY1-ME), a H_2O_2-selective fluorescent dye, then were exposed to H_2O_2 and analyzed by flow cytometry. Application of H_2O_2 resulted in a greater increase in the intracellular PY1-ME signal in hAQP3- or hAQP8-overexpressing HEK 293 cells than in control or hAQP1-expressing cells, confirming the results of the yeast studies and demonstrating that some members of the AQP family are capable of mediating the transmembrane diffusion of H_2O_2 into mammalian cells, while others do not [80]. In the same study, the facilitated uptake of H_2O_2 by hAQP3 was confirmed using the genetically encoded fluorescent H_2O_2 sensor, HyPer, which is able to detect very low levels of H_2O_2. In addition, to determine whether endogenously expressed hAQP3 could mediate H_2O_2 influx and downstream intracellular signaling, the authors used HT29 human colon cancer adenocarcinoma cells, which endogenously express hAQP3 and have transmembrane redox signaling pathways. Knock-down of endogenous hAQP3 expression using shRNA prevented H_2O_2 uptake, demonstrating that natural levels of hAQP3 regulate H_2O_2 influx into these cells. Treatment of HT29 cells with epidermal growth factor (EGF) resulted in activation of Nox proteins, extracellular NAD(P)H oxidases producing H_2O_2, which correlated with the intracellular phosphorylation status of the serine/threonine kinase AKT/protein kinase B, a key node in signal transduction [81]. Knock-down of hAQP3 expression induced a significant decrease in EGF-induced AKT phosphorylation, demonstrating that hAQP3-mediated uptake of naturally generated H_2O_2 also determines intracellular redox signaling [80]. Taken together, these data clearly demonstrated that extracellularly produced H_2O_2 is channeled via endogenous hAQP3 into the cell, where it activates downstream signaling cascades. This study provided the first evidence that AQPs play a physiologically important role in H_2O_2 transmembrane transport.

Hara-Chikuma et al. [82] confirmed the crucial role of AQP3 in the transmembrane transmission of H_2O_2 signals in another physiological context, that of chemokine-dependent T lymphocyte migration during the immune response in mice. These authors showed that the trafficking of $CD4^+$ T cells during cutaneous immune reactions was impaired in homozygous $AQP3^{-/-}$ knockout mice compared to wild-type cells, and that this cell migration process was dependent on AQP3-mediated H_2O_2 uptake, but not AQP3-mediated water transport. In response to chemotactic signals, T cells reorganize their actin cytoskeleton through the action of Rho family small GTPases, such as Cdc42, and become polarized, resulting in T cell trafficking [83]. Hara-Chikuma et al. [82] showed that the activation of Cdc42 and the subsequent actin changes required AQP3-mediated transport of H_2O_2, probably produced extracellularly by Nox NADPH oxidases after activation by chemokine ligands, including CXCL12, and that exogenous application of high levels of H_2O_2 rescued CXCL12-stimulated migration ability in $AQP3^{-/-}$ cells, probably due to increased passive uptake of H_2O_2. This signaling cascade therefore involves the transmembrane transport of extracellularly produced H_2O_2 into the cells. They also showed that, as in mouse T cells, silencing of endogenous hAQP3 in human T cells using siRNA resulted in impaired CXCL12-induced actin polymerization and that transfection of these cells with dominant active V12 Cdc42 cDNA restored actin polymerization [82]. Together, these studies, performed in mammalian cells, demonstrate that AQP3-mediated H_2O_2 uptake is important for the activation of intracellular downstream signaling events (Fig. 2).

3.3.5.2. Role of AQP8 in the transmembrane diffusion of hydrogen peroxide. Another study aimed at revealing the molecular mechanisms by which extracellularly produced H_2O_2 signals (e.g. by NAD(P)H oxidases) initiate intracellular signaling by phosphorylation of target proteins was undertaken by Bertolotti and co-workers [65]. Incubation of HeLa cells expressing the fluorescent H_2O_2 sensor HyPer with the AQP inhibitor $HgCl_2$ prevented the production of a fluorescent signal after application of external H_2O_2, suggesting that AQPs were responsible for the uptake of H_2O_2. Interestingly, silencing of hAQP8 prevented the increase in HyPer fluorescence after treatment with extracellular H_2O_2, demonstrating that this channel facilitated H_2O_2 diffusion into HeLa cells. When these authors expressed an AQP8 cDNA with five silent point mutations in the siRNA target sequence in the endogenous AQP8-silenced HeLa cells, H_2O_2 influx was restored. To determine whether hAQP8 was essential for the EGF-induced transient spike in cytosolic H_2O_2 and downstream signaling involving inhibition of phosphatases and activation of kinases, levels of tyrosine-phosphorylated proteins were measured. Silencing of AQP8 decreases the amount of tyrosine-phosphorylated proteins to a similar level as that seen when the H_2O_2-degrading enzyme catalase was added extracellularly. These results are in agreement with the hypothesis that hAQP8 allows uptake of extracellularly generated H_2O_2, amplifying the signal transduction pathway. Thus, hAQP8 silencing inhibits not only H_2O_2 uptake, but also intracellular downstream tyrosine phosphorylation of target proteins.

In addition to its plasma membrane localization, AQP8 is present in the inner membrane of mitochondria, which are a major source of ROS in animal and plant cells [84,85]. Marchissio and co-workers [72] examined whether knockdown of mitochondrial AQP8 (mtAQP8) in human hepatoma HepG2 cells using siRNA had an effect on mitochondrial H_2O_2 efflux and found that, compared to wild-type mitochondria, isolated mtAQP8 knockdown mitochondria had a lower H_2O_2 efflux ability, monitored using the Amplex Red/peroxidase system, and higher mitochondrial ROS levels, monitored using HDCFDA, and that permeabilization of the mitochondrial membrane with digitonin blocked this effect. The higher ROS levels in mtAQP8 knockdown cells resulted in a loss of cell viability, and the mitochondrially targeted antioxidant MitoTempol prevented ROS accumulation. These data from HepG2 human hepatoma cells suggest that mtAQP8 facilitates mitochondrial H_2O_2 release and that its defective expression may lead to ROS-induced mitochondrial dysfunction caused by the unspecific opening of the mitochondrial permeability transition pore and to cell death.

3.3.5.3. Role of AQP1 in the transmembrane diffusion of hydrogen peroxide. As stated above, human hAQP1 was found to lack H_2O_2 transport ability when tested using the yeast toxicity assay. The diffusion of H_2O_2 across erythrocyte membranes, in which hAQP1 represents the major AQP isoform, is insensitive to organic mercurials (typical hAQP1 blockers) [86], suggesting that hAQP1 does not significantly contribute to H_2O_2 transport in this cell type, and the results obtained with HEK 293 cells showing that hAQP1 does not transport H_2O_2 [80] are consistent with this.

In contrast to hAQP1, a physiological role of rat rAQP1, which is permeable to H_2O_2 in the yeast system (E. Beitz, personal communication), in regulating the H_2O_2 permeability of rat aortic smooth muscle cells (rASMCs) was recently suggested [87]. These authors showed that pathophysiological extracellular H_2O_2 levels caused an increase in Nox1-produced $O_2^{\bullet-}$ levels, subsequent Ask1 activation, and consequent smooth muscle cell hypertrophy. As rAQP1 is expressed in rASMCs, while the other known H_2O_2-permeable AQP3 and AQP8 isoforms are not, rAQP1 was hypothesized to be the cellular entry pathway for H_2O_2. To test whether rAQP1 facilitated the passive diffusion of H_2O_2

into rASMCs, the cells were co-transfected with non-functional or functional AQP1 siRNA and the genetically encoded H_2O_2 fluorescent probe HyPer. The results showed that addition of H_2O_2 to rAQP1-expressing rASMC resulted in a significant increase in intracellular HyPer fluorescence, indicating uptake of H_2O_2, and that this increase was not seen in AQP1-silenced cells. Together, these data show that rAQP1 mediates H_2O_2 entry into rASMCs and confirm the important physiological role of mammalian AQPs in transmembrane H_2O_2 fluxes.

*3.3.5.4. Physiological roles of plant AQPs in the facilitated transmembrane diffusion of hydrogen peroxide?*There are currently no data demonstrating a physiological role for a specific plant AQP in facilitating membrane diffusion of H_2O_2 in signaling cascades or detoxification processes, although such functions have been repeatedly suggested (see in [13,60,63,64,88]. A model of diffusion-mediated H_2O_2 signal propagation from the plasma membrane through the cytosol in a plant cell was recently proposed [88]. An important component of this model is that AQPs are considered to "emit" a cellular H_2O_2 signal consisting of short pulses produced by the rapid and synchronized opening and closing of the channels. The resulting signal amplitude will therefore depend on the density of the H_2O_2-permeable AQPs, the flux per channel, and the time they remain open. The experimental demonstration of this interesting hypothesis will be technically difficult. One explanation for the lack of knowledge about the physiological role of plant AQPs in H_2O_2 signaling might be the smaller number of available types of plant suspension culture cells compared to mammalian cell lines. Detailed information about transmembrane H_2O_2 signaling pathways in these plant cell lines, which could be used to investigate the impact of AQPs on H_2O_2 transport, is scarce compared to knowledge about mammalian signaling pathways. A further difficulty is the large number of AQP isoforms in plants (>30 in higher plants) compared to mammals (13 AQPs), which might result in functional redundancy in many cell types, preventing the elucidation of the functional role of a single isoform.

*3.3.5.5. Are AQPs essential for regulating hydrogen peroxide membrane diffusion in all organisms?*Although there is no physiological evidence for AQP-mediated H_2O_2 diffusion in plants, the importance of AQPs has been strongly suggested in experimental studies. In wild-type *Saccharomyces cerevisiae* cells, AQPs do not seem to play any important role in transmembrane transport of H_2O_2, since the H_2O_2 permeability constant is not altered in the presence of the AQP inhibitor $HgCl_2$ [58]. This result is in agreement with the results of Bienert et al. [13], who observed that wild-type BY4741 *S. cerevisiae* and the *Δfps1*, *Δyfl054c* mutant, which lacks all four of the yeast genome-encoded AQPs (*FPS1* and *YFL054c* genes are deleted and pAQY1 and pAQY2 are not functional in the genetic background of this mutant) [89,90], have a similar sensitivity to H_2O_2. These results suggest that neither pFPS1 nor pYFL054c is involved in H_2O_2 transport and support the notion that, in *S. cerevisiae*, H_2O_2 diffusion across the plasma membrane does not involve AQPs and that not all organisms use AQPs to regulate H_2O_2 membrane permeability.

3.3.6. Are plant AQPs gated by hydrogen peroxide?

H_2O_2 has not only been shown to be transported by AQPs, but also been suggested to be an inhibitor of plant AQPs. In *Tradescantia fluminensis*, H_2O_2 treatment of the leaf petiole resulted in a significant decrease in the water permeability of epidermal cells measured with a cell pressure probe [91]. Similarly, treatment with a combination of H_2O_2 and Fe^{2+} strongly decreases the water permeability of the parenchyma cells of maize leaves [92]. The redox inhibition of the water permeability of the plasma membrane was abolished using an antioxidant [93], suggesting that the inhibition of AQP activity is probably due to oxidative gating by ROS, such as •OH radicals, which are produced in the Fenton reaction. However, no inhibitory effect of H_2O_2 or t-butylhydroperoxide on the water channel activity of individual AQPs heterologously expressed in yeast or *Xenopus* oocytes has been demonstrated [13,14,94,95]. Several AQPs, including the H_2O_2-permeable PIPs, have redox-sensitive and structurally exposed cysteine residues that could act as sensors of the redox status. However, the impact on protein functionality of these residues in maize PIPs was recently tested in vivo in a mutational study and no redox-related function of these residues was identified [94]. Boursiac et al. [95] showed that ROS, including H_2O_2, do not gate PIPs through a direct oxidative mechanism, but act indirectly on the plasma membrane water permeability via PIP internalization into vesicles. The mechanisms by which H_2O_2 regulates the water permeability of the plasma membrane could involve different direct and indirect mechanisms, such as direct oxidative gating, the induction of signal transduction pathways leading to the internalization of AQP proteins, or the modification of their phosphorylation status, a post-translational modification regulating their gating and subcellular localization [96–98]. A study in which the hydraulic conductance of maize root was found to decrease when treated with H_2O_2 also suggests such indirect modification mechanisms, as the decrease in root hydraulic conductance was not accompanied by a significant change in levels of PIP1 and PIP2 proteins, but by increased phosphorylation of the PIP2s [99]. Interestingly, using an absolute quantification method, another study [100] showed that phosphorylation of *Arabidopsis* AtPIP2;1, an H_2O_2-permeable AQP [14], at two C-terminal sites (Ser280 and Ser283) was altered in response to H_2O_2 application, which, at the same time, markedly decreased root hydraulic conductance, and this changed phosphorylation pattern correlated with internalization of AtPIP2;1 into intracellular vesicles, thus explaining the decrease in root hydraulic conductance. It seems that signaling cascades that result in AQP post-translational modifications controlling their localization or gating are sufficiently fast enough to respond to abiotic stimuli such as oxidative stress.

These data suggest that alteration of AQP activity and localization in response to H_2O_2 may represent an efficient way of adjusting not only the transport of water and other substrates, but also, in a feedback loop, the transport of H_2O_2 itself (Fig. 2).

3.3.7. Is AQP expression regulated by hydrogen peroxide?

In addition to its impact on the gating and localization of AQPs, H_2O_2 can also alter their expression. Hooijmaijers et al. [71] examined the H_2O_2-regulated expression patterns of all 13 PIPs from *Arabidopsis*, and determined their permeability to H_2O_2 in a yeast toxicity growth assay and found that H_2O_2 treatment of *Arabidopsis* downregulated the expression of AtPIP2s in roots, but not in leaves, whereas expression of AtPIP1s was not affected. Interestingly, PIP1s do not facilitate the diffusion of H_2O_2 [14,68,71]. The growth and survival of yeast cells expressing AtPIP2;2, AtPIP2;4, AtPIP2;5, or AtPIP2;7, but not any other AtPIP, were reduced in the presence of H_2O_2 [71]. These results show that H_2O_2 regulates the expression of both H_2O_2-permeable and H_2O_2-impermeable AtPIPs and that AtPIP2;2, AtPIP2;4, AtPIP2;5, and AtPIP2;7 are channels for H_2O_2. The integrated regulation of AQP expression by H_2O_2 and the ability of individual AQPs to transport H_2O_2 might be important in plant responses to H_2O_2 (Fig. 2).

4. Conclusions

Evidence from studies on different organisms and using different experimental methods have highlighted the fact that specific AQP isoforms facilitate the passive diffusion of H_2O_2 across biological membranes and, therefore, crucially impact on the membrane permeability of H_2O_2. AQPs and their regulatory mechanisms can modulate intracellular and intercellular H_2O_2 fluxes and signaling in living organisms (Fig. 2). AQP-mediated transmembrane transport of H_2O_2 has been demonstrated to be of physiologically importance for further downstream signaling events, establishing the crucial contribution of AQPs in signaling processes in addition to their function in water homeostasis,

plant nutrition, and translocation of metabolically important or toxic substances.

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