Abstract

Rapidly accumulating evidence indicates that inflammatory T cells sensitively respond to their redox environment by activating signal transduction pathways. The hypothesis that T-cell receptors have the potential to catalytically transform singlet oxygen into H\textsubscript{2}O\textsubscript{2} attracted our attention since the biophysical regulation of this process would provide a new tool for therapeutically directing T cells down a preferred signaling pathway. Light-dependent production of H\textsubscript{2}O\textsubscript{2} was first described in antibodies, and we reproduced these findings. Using a real-time H\textsubscript{2}O\textsubscript{2} sensor we extended them by showing that the reaction proceeds in a biphasic way with a short-lived phase that is fast compared to the slow second phase of the reaction. We then showed that Jurkat T cells biophotonically produce about 30 nM H\textsubscript{2}O\textsubscript{2}/min/mg protein when pretreated with NaN\textsubscript{3}. This activity was concentrated 4 to 5 times in T-cell membrane preparations. The implications of these observations for the development of new therapeutic tools for inflammatory diseases are discussed.

Keywords: Reactive oxygen species; ROS; H\textsubscript{2}O\textsubscript{2}; O\textsubscript{2}; T lymphocytes; Real-time sensor; Ultraviolet light; Photosensitizer

T cells are central regulators of the body’s immune functions including inflammation. Inflammation is indispensable for survival, but excess inflammation is also the cause of debilitating diseases such as rheumatoid arthritis, heart disease, and some cancers. Treatments for these diseases are mainly aimed at limiting T-cell activity and thus preventing chronic inflammation. The most promising therapies for these kinds of diseases are those in which the cellular production of inflammation-causing substances such as autocrines and paracrines is controlled to direct the cells along a non-inflammatory metabolic pathway [1]. Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), a reactive oxygen species (ROS), is among the substances known to regulate T-cell inflammatory activity. A large body of evidence now indicates that T cells are able to sense H\textsubscript{2}O\textsubscript{2} and other ROS, and respond by activating transcription factors and associated signal transduction cascades that lead to inflammation [2,3]. Since the concentration and lifetime of ROS depend on the redox state of their environment, it is possible that significant changes in T-cell-based inflammatory processes might be achieved by modifying the redox state of T cells or their environment. So far, most studies on functions of ROS in T cells have been concerned with how cells detoxify ROS and thus reduce the unwanted effects of oxidative stress. However, it becomes more and more clear that inflammatory and non-inflammatory T cells also use ROS, especially H\textsubscript{2}O\textsubscript{2}, as intracellular and potentially intercellular messengers [4]. Since therapeutic regulation of inflammation could perhaps be accomplished by regulating H\textsubscript{2}O\textsubscript{2} metabolism in T cells, there is much interest in understanding the regulation of redox signaling in T cells. T cells are exposed to H\textsubscript{2}O\textsubscript{2} at sites of inflammation where activated phagocytes produce large amounts of ROS which are believed to protect against microbial infection. Phagocytic immune cells use NADPH oxidase to
reduce oxygen to superoxide, which is then enzymatically converted to \( \text{H}_2\text{O}_2 \). \( \text{H}_2\text{O}_2 \) can react with a newly formed superoxide and produce a hydroxyl radical, the main anti-bacterial agent. Previously it was believed that T cells could not produce \( \text{H}_2\text{O}_2 \), as phagocytic immune cells are known to do, due to their lack of the phagocyte-type NADPH oxidase. However, Jackson et al. [5] recently demonstrated that after stimulating the T-cell receptor (TCR), apoptotic signals including Fas ligand and Fas appeared, followed by NADPH oxidase activation and \( \text{H}_2\text{O}_2 \) production. Kinetic studies indicate that in addition to the sustained NADPH oxidase-dependent \( \text{H}_2\text{O}_2 \) production, there seems to be one or more other sources of \( \text{H}_2\text{O}_2 \) that transiently produce \( \text{H}_2\text{O}_2 \) independent of Fas or NADPH oxidase. One such potential source is the TCR itself. This idea stems from studies with purified antibodies and TCR molecules, which have the ability to catalyze a light-dependent biophotonic reaction between molecular oxygen and water that leads to the oxidation of water and the production of \( \text{H}_2\text{O}_2 \) [6–11].

The molecular aspects of the biophotonic water oxidation are most conveniently investigated using antibodies, since all antibodies exhibit a light-dependent and NADPH oxidase-independent \( \text{H}_2\text{O}_2 \) production, very similar to that found in TCR stimulated T cells. Datta et al. [12] have proposed that the catalytic sites in antibodies are at the interface of light (L) and heavy (H) chains (Fig. 1), more specifically at the interface of two Greek key domains, which are special anti-parallel tertiary protein structures. They determined that the TCR also has a set of juxtaposed Greek key domains on the \( \alpha \) and \( \beta \) chains that appear to produce a structural site that is similar to that found in antibodies (Fig. 1) and thus believe it to be the site for catalysis of water oxidation in TCRs. Although the in vitro evidence for water oxidation in antibodies and TCRs is striking, the physiological significance, while undoubtedly important, remains uncertain [10].

Hydrogen peroxide is believed to modulate the level of protein phosphorylation which is a critical determinant of a protein’s signal transduction activity. The level or extent of cellular protein phosphorylation and thus the metabolic character of cells is determined by the ratio of active phosphorylases to active phosphatases. \( \text{H}_2\text{O}_2 \) seems to inhibit protein tyrosine phosphatases, which leads to an increased proportion of phosphorylated proteins and activation of TCR-associated protein tyrosine kinases such as p56\( \kappa \)c and ZAP-70 [3,13]. This kind of phosphorylation cascade is typical of TCR signaling events, which then leads to the phosphorylation of numerous other proteins with eventually long-term metabolic consequences. The type of outcome may vary and it depends on the cell’s metabolic state when the signal was encountered. For instance, different cell states vary in their activity of co-stimulatory receptor-initiated pathways such as CD4 or CD28 pathways, which by themselves are also sensitive to redox changes [14,15]. How such complex ROS signaling networks are initiated and controlled is one of the great mysteries needing to be unraveled. It is the goal of this paper to contribute to the fast-growing body of evidence on T-cell redox signaling. We first present our confirmatory studies on the light-dependent \( \text{H}_2\text{O}_2 \) production by antibodies using our recently developed micro-photoreactor and \( \text{H}_2\text{O}_2 \) real-time sensor [16,17]. We then present the results of our studies on \( \text{H}_2\text{O}_2 \) production in T cells and T-cell membranes, and discuss the relationship between \( \text{H}_2\text{O}_2 \) production by antibodies and T cells. We further discuss the implications of these findings for the development of new therapeutic tools for inflammatory diseases.

**Materials and methods**

**Experimental overview.** Biomaterial (antibodies, T cells, and T-cell membranes) was suspended in 50–1000 \( \mu \)l phosphate-buffered saline (PBS), placed in closed quartz or borosilicate glass vessels, and irradiated for 1–24 h in photochemical reactors using ultraviolet (UV) or white light. The rate of \( \text{H}_2\text{O}_2 \) production was determined using the fluorometric assay Amplex Red (A-12222, Molecular Probes, Invitrogen, Carlsbad, CA) and our recently developed electrochemical real-time sensor. In some experiments, the samples contained 40 \( \mu \)M hematoporphyrin (H-5518, Sigma, St. Louis, MO) to enhance photoabsorption. Temperature within the reaction vessel was monitored using digital and mercury thermometers, and remained constant at 37 ± 0.5 °C. As positive controls, 100 \( \mu \)M \( \text{H}_2\text{O}_2 \) in PBS was prepared from a stabilized 30% (w/w) stock solution (H-1009, Sigma, St. Louis, MO) and processed in parallel with the biomaterial samples. For \( \text{H}_2\text{O}_2 \) dismutation, 2.5 U of catalase (C-9322, Sigma, St. Louis, MO) was added per 50 \( \mu \)l of sample. As negative controls, samples with PBS alone and biomaterial samples shielded from light were used. For shielding, we either coated the vessels with a black Krylon paint, or, covered them with a plastic straw. Additional control samples included 6.7 \( \mu \)M albumin (A-7888), myoglobin (M-1882), and aprotinin (A-6279, Sigma, St. Louis, MO). \( \beta \)-Microglobulin (PHF 135) was from Serotec (Raleigh, NC). Since these proteins lack the structural sites that are proposed as catalytic water oxidation sites in antibodies and TCRs, results using these proteins can give some insight into the specificity of \( \text{H}_2\text{O}_2 \) production by antibodies and T cells.
Biomaterials (antibodies, T cells, and T-cell membranes). To detect light-dependent H$_2$O$_2$ production by antibodies, ChromoPure whole rat IgG (JOR000003) was purchased from Accurate Chemical and Scientific (Westbury, NY). Human monoclonal anti-CD3, purified from the growth medium of OKT3 cultures (ATCCC, CRL 8001), was a gift from Mary T. Johnson (Indiana University School of Medicine).

Jurkat cells, TIB 152, clone E6.1, a human T lymphocyte cell line, were purchased from the American Type Cell Culture (Rockville, MD). Cells were cultured in RPMI 1640 plus 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, and 0.29 mg/ml l-glutamine, at 37 °C in a humidified 5% CO$_2$/95% air atmosphere. Cells were counted with a hemocytometer and cell viability was determined by trypan blue exclusion.

Crude extracts were prepared from 10$^3$ to 10$^4$ Jurkat cells using a modified protocol from Morre and Morre [18]. Briefly, cells were washed in 0.25% M sucrose and 0.5 mM Hepes, pH 7.5, and resuspended in ice-cold buffer of 0.125 M sucrose, 0.25 mM Hepes, and 200 µg/ml DNase (D-8764, Sigma, St. Louis, MO). Cells were homogenized using a Teflon tissue grinder and lysed by adding chilled distilled H$_2$O, resulting in greater than 90% cell breakage as determined microscopically. Homogenate was centrifuged at 500g for 15 min to remove unbroken cells and nuclei. Supernatant containing plasma membranes was centrifuged at 10,000g for 30 min. Pellets were treated with 1 mM NaN$_3$ (2352, Eastman Kodak, Rochester, NY) for 10 min to inhibit interfering endogenous catalase activity. Excess NaN$_3$ was removed by washing the samples three times with distilled H$_2$O. Protein concentration was determined using the BCA protein assay including albumin standards (23227, Pierce Biotechnology, Rockford, IL). Membranes were frozen in aliquots at −80 °C until used in experiments at a concentration of 1 mg/ml.

H$_2$O$_2$ detection (Amplex Red, real-time sensor). After exposure to light, aliquots of experimental samples and controls (see Experimental overview) were tested for H$_2$O$_2$ content by incubating them for 30 min at room temperature in 0.1 M sodium phosphate, pH 7.4, containing 50 µM Amplex Red and 5 U/ml horseradish peroxidase (HRP, P-2088, Sigma, St. Louis, MO). Amplex Red is oxidized to resorufin in the presence of H$_2$O$_2$ and HRP. Resorufin was quantitated spectrophotometrically using a BioTek EL 808 multimwell plate reader. For real-time detection of H$_2$O$_2$ production, a Clark oxygen electrode was polarized at 0.7 V at the platinum electrode relative to the silver/silver chloride electrode and used with sample volumes of 0.5 ml or greater. For smaller volume assays such as when testing plasma membrane preparations, a D-1089-02 bipolar heart pacing catheter tipped electrode (Webster Labs, Altadena, CA) was used. To avoid deactivating the electrode surface, the electrodes were protected with a polyurethane hydrophilic Gelnots membrane (Tomen America, New York, NY). To record output voltage of the electrode, a high gain amplifier consisting of a current to voltage converter and an amplifier circuit was used. Data were collected using a Multilog 720 multimeter and software (Estech Instruments, Waltham, MA) coupled to a PC, or, using the Agilent data acquisition hardware (34970A) and corresponding Benchlink software.

Photocatalytic reactors. To detect H$_2$O$_2$ production in large volume samples (0.5–3 ml), samples were placed in a thermoregulated Lucite reaction vessel with a quartz window for light irradiation. The temperature of the reaction vessel was maintained at 37 ± 0.5 °C with the aid of a constant temperature, Savant Model RWC-825 circulating water bath.

We developed a simple photochemical microreactor for small (50 µl) volume samples. The reactor consists of a lamp mounted in the center of the reactor and 12 samples circumferentially arranged around it at about 1 cm from the lamp [19]. As white light source we used a F8T5 CW fluorescent tube from the General Electric Company. Using a DLM2 light meter from Sherman Instruments (Vancouver, British Columbia, Canada) we estimate that 50 µl samples are exposed to 140 lux, which translates to 50 nJ/min at 555 nm using again the 4 mm$^2$ as estimate of the irradiated sample surface.

Results

H$_2$O$_2$ production by antibodies

For several years our laboratory has been interested in studying the biological action of the TCR and when Wentworth et al. reported that antibodies and TCRs appeared to catalyze the biophotonic conversion of water to H$_2$O$_2$ we set out to investigate this phenomenon in conjunction with our other interests. As a prelude to studies on the physiological implications of this phenomenon, we proceeded to reproduce the early results of these authors. Using the same assay for detection of H$_2$O$_2$ that Wentworth et al. employed, the Amplex Red assay, we found that 50 µl of reaction mixture containing 6.7 µM rat IgG produced about 57 nM H$_2$O$_2$/min (Fig. 2, column 1) or a specific rate of 0.06 nmol H$_2$O$_2$/min/mg protein when irradiated with 300 µW/cm$^2$ UV light. This translates into a light energy of 720 µJ/min or 2 × 10$^{13}$ photons at 312 nm and 4 mm$^2$ as an estimate of the surface area irradiated. Assuming 100% photoefficiency the antibody molecules produced about 30 molecules of H$_2$O$_2$ per photon. In eight independent replicate experiments, this rate varied by about 15% (data not shown). We attribute the ±15% experimental variation in the H$_2$O$_2$ production rate to variation in the light flux that was used to excite the antibodies in the different experiments. The rate of H$_2$O$_2$ production was constant over several hours accumulating to about 83 µM H$_2$O$_2$ in 24 h (Fig. 2, inset).
production rate of β2-microglobulin, a protein with one Greek key motif, we found a H$_2$O$_2$ production rate of about 10–15% of that obtained with antibody (data not shown). We therefore agree with Datta et al.[12] that juxtaposed Greek key domains might be important for light-catalyzed H$_2$O$_2$ production by proteins. H$_2$O$_2$ production by various non-antibody proteins, all prepared at 6.7 µM in PBS, was 80–90% less than that produced by the same concentration of antibody.

As illustrated in Fig. 2, column 7, H$_2$O$_2$ production of UV-irradiated rat IgG increased about 4-fold when the antibodies were irradiated in the presence of hematoporphyrin (HP). HP is a photosensitizing agent which, like UV light, is known to generate singlet oxygen. HP alone did not cause any H$_2$O$_2$ production (Fig. 2, column 8). To determine if H$_2$O$_2$ production is solely dependent on UV light, we irradiated antibodies with white light. As shown in Fig. 2, columns 9–14, we did not detect any H$_2$O$_2$ production in the 50 µl rat IgG samples when irradiated with 140 lux white light. However, when antibodies were irradiated with white light in the presence of HP we measured about 85 nM/min H$_2$O$_2$ production. Although HP has its strongest absorbance between 380 and 420 nm, it does absorb visible light to create singlet oxygen [20], a process employed in photodynamic therapy [21]. Control samples (antibody + HP shielded from white light; antibody + HP + catalase; HP, and PBS alone) did not produce measurable quantities of H$_2$O$_2$. In general, we obtained results similar to those reported by Wentworth et al. using similar proteins and equipment.

The fluorescence assay for H$_2$O$_2$ using Amplex Red is sensitive and convenient but the method is not readily amenable to real-time monitoring. Consequently, we developed a system including a H$_2$O$_2$ fixed platinum silver/silver chloride polarography electrode to study biological H$_2$O$_2$ production in real time. The system is described in more detail elsewhere [16,17]. With the platinum anode at the optimized polarizing voltage of +0.7 Volts, the electrode output signal is proportional to H$_2$O$_2$ concentrations between 0 and 100 µM. In the experiment illustrated in Fig. 3, 9 µM CD3 antibody was irradiated with UV light through the quartz window of a water-jacketed reaction vessel whose contents were stirred with a magnetic stirrer. The reaction temperature was maintained at 37 ± 0.2°C using a circulating water bath. After thermal equilibration of the system (not shown), a linear increase in H$_2$O$_2$ content of the reaction mixture was detected with a rate of 1.375 mV/min for about 20 min, which translates after calibration into about 550 nM H$_2$O$_2$/min. The signal output from the electrode was confirmed to be H$_2$O$_2$ by adding catalase, which catalyzed the degradation of H$_2$O$_2$ and returned the electrode current to its baseline level.
contents were stirred with a magnetic stirrer. The reaction temperature was maintained at 37 °C using a circulating water bath. After thermal equilibration of the system (not shown), we detected a UV-dependent, linear increase in H$_2$O$_2$ content of the reaction mixture with a rate of 1.375 mV/min for about 30 min, which translates into about 550 nM H$_2$O$_2$/min. The H$_2$O$_2$ production continued for 24 h, but became non-linear at higher H$_2$O$_2$ concentrations. The signal output from the electrode was confirmed to be H$_2$O$_2$ by adding catalase, which catalyzed the degradation of H$_2$O$_2$ and returned the electrode current to its baseline level.

H$_2$O$_2$ production by T cells and T-cell membranes

The TCR is known to contain protein sequences similar to the sequences of proposed proteins that make up the proposed catalytic sites for biophotonic water oxidation in antibodies and it was shown that purified γδ TCR also photochemically produce H$_2$O$_2$[6,7]. Based on this we anticipated that we should be able to detect H$_2$O$_2$ production by T cells and T-cell membranes using our real-time sensor. To test this expectation, we produced membrane preparations from Jurkat cells, a T lymphocyte cell line. Fig. 4 shows that, like antibodies, membrane preparations from 10$^7$ cells irradiated with UV light catalyzed a robust H$_2$O$_2$ production over the 20-h observation period. The rate of voltage change was initially linear and translates into about 130 nM H$_2$O$_2$/min/mg protein, but like the results with antibodies the rate of H$_2$O$_2$ production became non-linear at higher H$_2$O$_2$ concentrations. Near the termination of the experiment shown in Fig. 4 a calibrating quantity of H$_2$O$_2$ was added to the reaction mixture resulting in a sharp increase in electrode signal. When catalase was subsequently added to the reaction vessel at the end of the experiment, the electrode signal returned to baseline as the biologically produced and the calibrating H$_2$O$_2$ were dismutated. The production of H$_2$O$_2$ measured in real time was confirmed at 500 and at 1150 min by removing aliquots of reaction mixture and assaying the H$_2$O$_2$ content using Amplex Red. Control samples comprised of T-cell membranes without UV irradiation (not shown), and buffer or samples containing bovine serum albumin irradiated with UV (Fig. 4, inset) did not generate an electrode signal. These data indicate that T-cell membranes produce H$_2$O$_2$ in a membrane-associated, light-dependent event.

Next, we tested whether the light-dependent H$_2$O$_2$ production of cell membrane preparations can also be measured in whole cells. Using the H$_2$O$_2$ electrode we found that normal Jurkat cells at a density of 10$^7$ cells/ml produced about 65 nM H$_2$O$_2$/min, which again was sensitive to degradation by catalase (Fig. 5). However, the production of H$_2$O$_2$ by intact T cells could only be measured when

![Fig. 4](image_url) Polarographic real-time detection of the biophotonic H$_2$O$_2$ production by Jurkat T-cell membranes. Membranes were prepared from 10$^7$ cells, irradiated with UV light (312 nm maximal emission), and the rate of voltage change was monitored for 20 h. The initial rate translates into about 130 nM H$_2$O$_2$/min/mg protein. At 500 and 1150 min H$_2$O$_2$ was additionally assayed using Amplex Red (AR). At 1200 min a calibrating quantity of H$_2$O$_2$ was added resulting in a sharp increase of the electrode signal. When catalase was added, the signal returned to baseline as the biologically produced and the calibrating H$_2$O$_2$ was dismutated. Samples with bovine serum albumin did not generate an electrode signal when irradiated with UV light (inset).

Fig. 5. Polarographic real-time detection of the biophotonic H$_2$O$_2$ production by Jurkat T cells. 10$^7$ cells pretreated with sodium azide were suspended in 1 ml of phosphate-buffered saline and exposed to UV light (312 nm maximal emission). The rate of voltage change was monitored for 20 h and translates into about 65 nM H$_2$O$_2$/min after calibration. Addition of H$_2$O$_2$ dismutating catalase made the signal return to baseline.

![Fig. 6](image_url) Polarographic real-time detection of H$_2$O$_2$ dismutation by normal Jurkat cells (left) and Jurkat cells washed with sodium azide (right). At the indicated time, a bolus of 30 μM H$_2$O$_2$ was added to Jurkat cells at a concentration of 2 x 10$^5$ cells/ml. H$_2$O$_2$ rapidly disappeared from the culture following first-order kinetics. This H$_2$O$_2$ dismutating activity was inhibited by azide.
the cells were pretreated with sodium azide to inhibit their abundant stores of endogenous catalase and other H$_2$O$_2$ dismutases. Fig. 6 illustrates that untreated Jurkat T cells can rapidly dismutate 30 μM H$_2$O$_2$ with first-order kinetics and it shows that this activity is inhibited by azide.

**Discussion**

Like Wentworth and colleagues we have shown that the production of H$_2$O$_2$ by all tested antibodies is mediated via singlet oxygen that reacts with water to produce H$_2$O$_2$. We have extended these observations by observing that the reaction proceeds by a biphasic reaction process. With our polarographic H$_2$O$_2$ assay system we obtained the first real-time picture of antibody catalyzed biophotonic H$_2$O$_2$ production. We observed that human monoclonal anti-CD3 had an initial rate of about 400 nM H$_2$O$_2$/min/mg production. We observed that human monoclonal anti-CD3 had an initial rate of about 400 nM H$_2$O$_2$/min/mg protein when irradiated with UV light of 312 nm. This first, short-lived phase of the reaction is very fast compared to the slow second phase of the reaction. Quantitative comparisons between our H$_2$O$_2$ production rates and the rates reported by Wentworth et al. are problematic without exact comparisons of the properties of the photoreactors used in the studies since the rate of H$_2$O$_2$ biosynthesis exhibits a marked dependency on the illumination intensity.

A wide variety of non-antibody proteins have been shown to catalyze H$_2$O$_2$ production, most with much lower rates of water oxidation. However, purified αβ TCRs oxidize water with an efficiency approaching that of antibodies. The similarity in water oxidation activity of antibodies and TCRs has been attributed to the presence of a characteristic H$_2$O$_2$ producing catalytic site in both kinds of molecules [12]. Although it is well known that T cells can sense and respond to H$_2$O$_2$, it has only more recently been proposed that T cells themselves produce H$_2$O$_2$ [22,23]. The H$_2$O$_2$ from T cells does not seem to be derived from superoxide anions as believed to be the case in many other systems where the pathway from molecular oxygen to H$_2$O$_2$ proceeds with superoxide as an intermediate. Combining the proposal of a superoxide-independent H$_2$O$_2$ production in T cells with the known superoxide-independent ability of purified TCR proteins to oxidize water, we hypothesized that the production of H$_2$O$_2$ by TCRs in vivo plays an important role in T-cell redox signaling. To test this hypothesis, we developed a polarographic method to measure H$_2$O$_2$ in real time [16,17] and a micro-photoreactor, which can be used to study multiple 50 μl samples simultaneously [19]. We used these two new tools to define the characteristics of H$_2$O$_2$ production by Jurkat cells, a T lymphocyte cell line, and a whole cell membrane preparation derived from Jurkat cells.

We show for the first time a light-dependent H$_2$O$_2$ production by intact T cells washed with sodium azide to inhibit enzymic H$_2$O$_2$ dismutating activity. In order to determine if the H$_2$O$_2$ production by intact Jurkat cells was associated with the membrane localized TCR we prepared a crude water and azide washed Jurkat cell membrane preparation in which the catalase-like cell activities are mostly removed and the remaining catalase activity is inhibited by the azide. Membrane preparations from about 10$^7$ cells biophotonically produced about 130 nM H$_2$O$_2$/min/mg protein when irradiated with UV light. We calculated this membrane-associated activity to be four to five times more concentrated in water oxidation activity compared to intact cells. This suggests that the activity is in fact associated with the membrane localized TCRs.

There are of course other H$_2$O$_2$ producing activities in cells besides the TCR, but they are not known to be activated by light or singlet oxygen like that presumed for the TCR. Two of the more well-known H$_2$O$_2$ producing activities are the cell membrane localized NADPH oxidase and the mitochondrial electron transport chain, but based on the following arguments neither is likely to substantially contribute to H$_2$O$_2$ production in our T-cell membrane preparations. First, activation of cell-membrane localized NADPH oxidase requires recruitment of several cytoplasmic proteins [5], which are almost certainly removed in our Jurkat membrane preparations as a consequence of the repetitive wash steps during the preparations. Second, even though it is possible, and even likely, that our membrane preparations are contaminated with mitochondrial membranes, the extensive washing removes most small organic cell molecules including Krebs cycle intermediates, pyridine nucleotides, and allied water soluble molecules, which are required to drive mitochondrial electron transport and to generate parasitic oxygen reduction related to electron transport activity. Future experiments are planned to confirm these arguments.

Jurkat T cells are known to have the capability of dismutating large amounts of H$_2$O$_2$ [24]. Here, we have reported that unless catalase-like cell activities are inhibited (e.g., by sodium azide) we cannot observe any accumulation of H$_2$O$_2$ with intact cells. These facts raise the question of the physiological significance of cell-based H$_2$O$_2$ production regardless of its mechanism of formation. Wentworth’s group has speculated that the primary role of water oxidation by antibodies, and by extension TCRs, might be a physiologically significant mechanism for destroying singlet oxygen. During inflammation, T cells are exposed to singlet oxygen produced by inflammatory leukocytes, and there is abundant literature on the harmful effects of singlet oxygen-induced stress on T cells [2,23]. Hence, it is possible that the oxidation of water is a way to protect the organism against the known toxic effects of singlet oxygen, rather than to provide a physiologically useful source of H$_2$O$_2$.

The TCR-based, light-driven conversion of singlet oxygen to H$_2$O$_2$ and then H$_2$O$_2$ may involve a mechanism in which a primed process has evolved for efficient, short-term, rapid elimination of singlet oxygen produced close to the T-cell membrane. The notion that components of the immune system might be primed to destroy a burst of singlet oxygen stems from observations uniquely made with our H$_2$O$_2$ electrode monitoring system in which we
observe a burst of H$_2$O$_2$ formation immediately upon illuminating T cells, membranes, or antibodies. This is followed by a marked decrease in H$_2$O$_2$ production with time. We estimate that in the first few minutes of the reaction the rate is about 50-fold greater than that observed after 30 min. It might be thought that our observations could be explained by loss of catalytic H$_2$O$_2$ producing activity. However, Wentworth et al. [6] have observed the same time-dependent change in water oxidation and they have shown that it is not due to irreversible loss of catalytic activity but that when the accumulated H$_2$O$_2$ is discharged relatively fast, H$_2$O$_2$ production resumes. We also observe these kinds of events, and thus, the light driven chemical reactions taking place seem to be of no consequence to the catalytic health of the process.

A second explanation for the decreasing H$_2$O$_2$ production with time might be that substrates (reactants) for the H$_2$O$_2$ producing reaction become limiting and thus slow the reaction. One of the substrates is singlet oxygen, but it seems not likely to become rate limiting over the course of the reaction. Since illumination of the reaction is not significantly different over the course of the reaction and since a vast atmospheric store of oxygen is available to replenish that lost by conversion to singlet oxygen, it is reasonable to assume that the rate of singlet oxygen production is effectively constant in our system. A second reactant is water. Water, in physiological fluids, including in our reaction system, has a concentration of about 55 M and thus cannot reasonably be suspected of becoming rate limiting.

One possible explanation for the fast initial activity followed by a slower H$_2$O$_2$ production is that an already primed catalytic site reacts with singlet oxygen when the light is turned on and that the re-priming process is slow. Since light and thus singlet oxygen are not present before the reaction starts, it is unlikely that the priming process would involve either light or singlet oxygen. The other primer candidate is water. Water is present in abundance and a necessary reactant for H$_2$O$_2$ production. Datta et al. [12] have identified key water binding sites buried deep within the antibody molecule and proposed a tortuous path for entry to the binding site(s). Thus, it is reasonable to propose that before singlet oxygen appears the water binding sites are saturated with water. After illumination, reaction of the resultant singlet oxygen with priming water at the water-saturated catalytic site leads to an initial fast rate, or burst, of H$_2$O$_2$ production. This burst depletes water at the catalytic site and limits the rate of the overall reaction, which subsequently depends on the rate of H$_2$O$_2$ diffusion away from the catalytic site and its replacement by water. Another, kinetic, way of looking at this process is that H$_2$O$_2$ accumulation in all of our experimental systems leads to product inhibition because of a significantly large back-reaction rate constant.

In our view, regardless of the mechanism involved in slowing the reaction, the result is that when singlet oxygen appears the system is primed for converting it to H$_2$O$_2$ and that the primed state is short lived. Physiologically, the availability of a primed mechanism for detoxifying the organism of singlet oxygen seems reasonable. In an organism, conditions for producing singlet oxygen are likely to appear in bursts. For example, respiratory burst is the release of reactive oxygen species including singlet oxygen from phagocytic immune cells such as neutrophils and macrophages during an immune response. Additionally, sudden exposures to high levels of singlet oxygen generated by solar light are daily occurrences. An exercise-induced or disease-initiated period of anaerobiosis followed by sudden oxygen reperfusion also generates singlet oxygen. Thus, the evolution of a plethora of mechanisms to protect against the appearance of bursts of singlet oxygen should not be unexpected. However, although H$_2$O$_2$ is much less reactive than singlet oxygen, it has the marked potential for forming hydroxyl radicals, which are likely to be even more damaging to biological material than singlet oxygen. As a consequence, organisms including Jurkat cells developed many tools to detoxify H$_2$O$_2$.

While cellular detoxification might be one evolutionary force for developing a process of immune system-based, catalytic water oxidation, it seems clear that evolutionary processes have turned the appearance of TCR generated H$_2$O$_2$ (as well as other ROS) into an advantage. In studies where H$_2$O$_2$ was added to T cells as a bolus, it was shown that like conventional organic ligands, H$_2$O$_2$ activates the TCR and a number of TCR-initiated signal transduction pathways [15,25]. It is possible that a second physiologically significant consequence of water oxidation is that the small quantities of H$_2$O$_2$ produced at the TCR are sufficient to activate the TCR, leading to TCR signaling. Extra H$_2$O$_2$ that is not used for signaling diffuses away from the TCR and is then readily dismutated by catalase and glutathione peroxidase, forming predictable H$_2$O$_2$ gradients across the cytoplasm and biomembranes [26].

Lastly, when discussing the potential physiological role of singlet oxygen-initiated redox processes by antibodies and T cells, the distribution and activation of natural photosensitizers need to be considered. Like Wentworth et al. [6], we found that the photosensitizer hematoporphyrin significantly enhances light-dependent water oxidation. In vivo, porphyrins are normal metabolic intermediates that are relatively insoluble in biological fluids and are normally present in relatively small quantities as a consequence of the normal turnover of hemoproteins such as hemoglobin. However, in pathological situations, where hemoglobin breakdown products accumulate (e.g., bruises) larger quantities of porphyrin can be found. It is thus possible that the biophotonic production of singlet oxygen in the circulation or at localized sites of HP deposition could contribute to a physiologically significant production of H$_2$O$_2$. Since our laboratory is interested in developing innovative therapies for T-cell-based inflammatory diseases, we will continue studying the relationship between singlet oxygen, H$_2$O$_2$, and T-cell signaling.
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