Chapter 5
Cellular Damage

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Abstract Classical pharmacology is normally concerned with defined molecular structures that can bind to specific proteins and either inhibit or enhance the protein function to achieve some biological response with therapeutic benefit. In photodynamic therapy (PDT) context, we rarely rely on such target specificity to achieve therapeutic success. Although some recent photosensitizers have been functionalized with target-specific molecules, such as antibodies, to recognize specific cells and enhance therapy specificity, ROS produced inside the cell will damage all susceptible molecules within the diffusion radius. According to the previous chapter, both hydroxyl radicals and singlet oxygen are highly reactive toward most of the abundant biological molecules contained in cells. In this chapter we discuss how such capacity of PDT to provoke multiple sites of molecular damages in the cellular context is associated with the phototoxicity produced. Also, we discuss how cellular antioxidant and xenobiotic defenses can influence on cellular tolerance against photodynamic inactivation.

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5.1 Introduction

Since the publication of the book entitled *Pathology of Oxygen*, written by A. Autor in 1982, the systemic biological effects caused by oxidative stress, including cellular damage and signaling events, have raised enough attention to become a new "hot topic" in biological sciences [1]. Indeed, following the development and refinement of reactive oxygen species (ROS) detection techniques during the 1970s it became possible to observe increased levels of free radicals associated with several health disorders such as cancer, neurodegenerative diseases, chronic inflammation, and ischemia and reperfusion-related injuries. Even today, the exact mechanisms to explain these observations concerning the various redox imbalances associated with diverse pathologies have yet to be completely elucidated, and redox biology is still a field of intensely active research.

Current scientific thinking no longer exclusively interprets ROS as sources of cellular damage [2]. Examined through an evolutionary perspective, it is logical that ROS should also play an important role as signaling molecules to regulate the metabolism of aerobic organisms. For example, the electron transport chain of cellular aerobic metabolism loses about 1 % of all transported electrons that go on to produce superoxide radicals; moreover, the phagocytic cells of our immune system use oxidative bursts that yield large amounts of ROS to inactivate pathogens. ROS are used for the synthesis of important metabolites, such as purine nucleotides and prostaglandins, where singlet oxygen seems to be a necessary reactant. More generally, most molecules present in the external environment are in oxidized forms, while in living organisms, most molecules can only function properly when reduced. Therefore, the participation of ROS in cell signaling pathways is strictly required to regulate the respiratory metabolic function and balance the redox state of cellular components.

The advent of bioinformatics and systems biology such as genomic, proteomic, and metabolomic studies has consistently corroborated these theories [3–5]. Antioxidant enzymes and ROS scavenger synthesis pathways that are responsible for redox regulation and defense against environmental sources of ROS are extremely well conserved throughout evolution (e.g., catalase, superoxide dismutase, glutathione) [5]. Conversely, gene expression regulated by oxidation-sensitive enzymes and transcription factors (e.g., IkB, HIF-1, oxyR) has been shown to govern vital cellular functions such as production of antioxidants, stage of cell cycle, inflammatory response, proliferation, motility, apoptotic signaling, and many others [2, 6]. Hence, contemporary scientific opinion offers an alternative to the old-fashioned idea that ROS were always harmful or contests the excessive simplicity of this viewpoint.

A good example of the possible dual nature of ROS is related to the practice of physical exercise. Brief bursts of metabolic activity during aerobic exercise yield moderate and short-lived amounts of ROS that can stimulate cells to upregulate antioxidant synthesis and cell growth pathways leading to protection against future oxidative insults, while exercise simultaneously induces muscle hypertrophy and

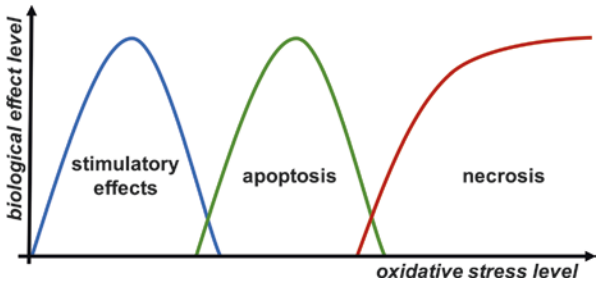

Fig. 5.1 Illustrative plot of biological effects induced by oxidative stress. In simple terms, low levels of oxidative stress stimulate cell metabolism, intermediary levels induce apoptosis signaling, and high levels can lead to direct necrosis. Autophagy is considered as an extreme survival strategy that occurs in oxidative stress levels where apoptosis and stimulatory effects overlap. Hence, autophagic cells may end up surviving or dying depending on a variety of factors

proliferation. On the other hand, if oxidative stress or physical exercise is too intense or too prolonged, damage caused by ROS may be sufficient to induce harmful effects such as inflammation, cell death, and tissue injury. Hence, the balance between beneficial and detrimental effects of ROS relies on its production site, rate, duration, and total yield (Fig. 5.1). Also, the balance between apoptotic and necrotic cell death pathways induced by ROS damage depends directly on the level of oxidative stress along with other factors such as the PS localization.

Classical pharmacology is normally concerned with defined molecular structures that can bind to specific proteins and either inhibit or enhance the protein function to achieve some biological response with therapeutic benefit. Hence, the molecular targets and mechanisms of action of most chemotherapy drugs can be precisely determined, and side effects are usually linked to lack of drug specificity. In PDT context, we rarely rely on such molecular target specificity to achieve therapeutic success. Although some recent photosensitizers have been functionalized with target-specific molecules, such as antibodies, to recognize specific cells and enhance therapy specificity, ROS produced inside the cell will damage all susceptible molecules within the diffusion radius. According to the previous chapter, both hydroxyl radicals and singlet oxygen are highly reactive toward most of the abundant biological molecules contained in cells. In this chapter we discuss how such molecular damages in the cellular context are associated with phototoxicity produced by PDT.

5.2 Ultrastructural Damage: How Does ROS Kill Cells?

Highly reactive oxygen species generated by photodynamic reactions, such as hydroxyl radicals (HO•) and singlet oxygen ($^1O_2$), can rapidly react with all molecular structures that are involved in the maintenance of proper cell physiology and viability. As described over the previous chapter, type 1 and type 2 reaction products can modify components of nucleic acids, proteins, and cell membranes inducing

Table 5.1 Reaction rate constants (k, L mol^{-1}s^{-1}) for hydroxyl radicals (HO$^{\bullet}$) and singlet oxygen ($^1O_2$) with some examples of relevant biomolecules. Proteins and DNA rate constants are based on human-derived macromolecules

Target	HO$^{\bullet}$	$^1O_2$
DNA	8×10^8	5.1×10^5
dG	7.6×10^9	5.3×10^6
dA	4×10^9	$<1\times10^5$
Albumin	7.8×10^{10}	5×10^8
Trypsin	1.6×10^{11}	8×10^9
Lysozyme	5.1×10^{10}	1.3×10^8
Histidine	1.3×10^{10}	9×10^7
Tryptophan	1.3×10^{10}	6.6×10^7
Cysteine	3.4×10^{10}	8.3×10^6
Methionine	7×10^9	8.6×10^6
Glycine	6×10^6	$<1\times10^5$
Arginine	3.5×10^9	–
Leucine	1.7×10^9	–
Linoleic acid	9×10^9	1×10^7
Glucose	1×10^{10}	1.4×10^4
Ascorbate	1×10^{10}	8.3×10^6
GSH	1.4×10^{10}	2.4×10^6

Data was obtained from literature publications [7, 8]

either reversible or irreversible damage. If sufficient damage were selectively delivered to any of those structures, it would already be enough to completely inactivate any cell. However, when dealing with highly reactive oxidants, and especially with HO•, we must expect to observe a ubiquitous pattern of widespread cellular damage. In Table 5.1 we show the reaction rate constants of HO• and $^1O_2$ with some examples of the most important biomolecules present in membranes, nucleic acids, saccharides, and proteins. Even though singlet oxygen and peroxides are more selective in the targets they react with, all biomolecular structures mentioned above are susceptible to oxidation in some specific positions.

The diffusion radius of highly reactive species tends to be very small in biological samples. Therefore, ROS react with the biomolecules closest to their production site, i.e., the region of photosensitizer accumulation. The overall reaction rate is determined by the reaction rate constant multiplied by the target concentration. Since the PS concentration varies among different compounds (Figs. 5.2a–c) and cell types, and even among cellular regions or compartments, the actual oxidative impact of PDT on each cellular biomolecule is most strongly influenced by PS concentration at the ROS production site. Hence, the amount of damage distributed to each cellular structure mostly depends on where the photosensitizer accumulates, the concentration of targets at that region, the rate constants for the reaction between oxidants and targets, the concentration of antioxidants, and the cellular capacity to repair oxidative damage. Based on all these factors, computer simulations suggest that the oxidant reactivity alone is insufficient to determine which cellular targets

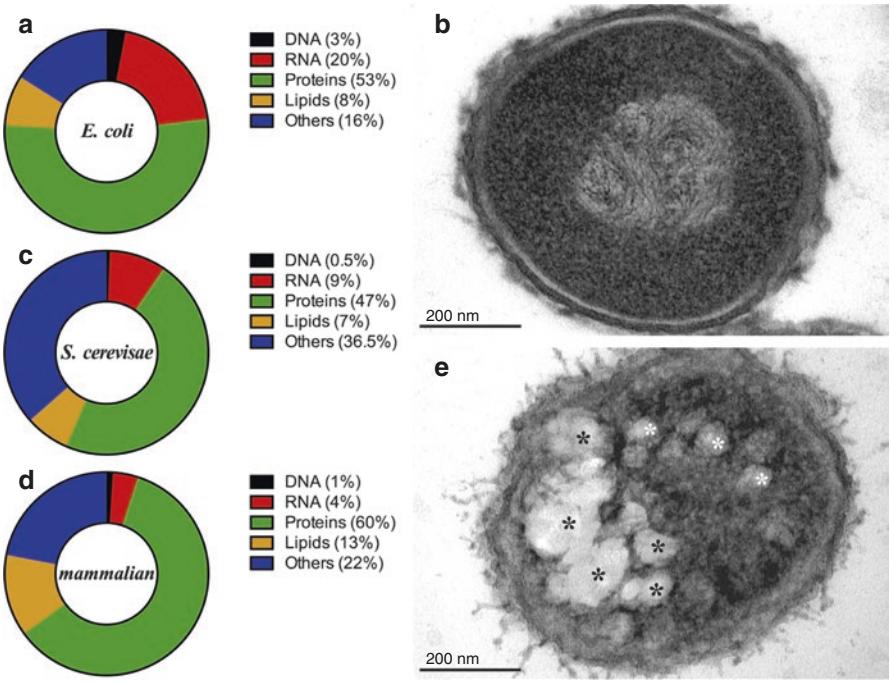

Fig. 5.2 From (**a**–**c**) we present the most abundant biomolecules present in *Escherichia coli* (**a**), *Saccharomyces cerevisiae*, (**b**) and human leukocytes (**c**) (Data from Feijo Delgado et al. [38]). Note that proteins are the major components of cells. Transmission electron microscopy images of transversal sections of a Gram-negative bacterium (*Klebsiella pneumoniae*) before (**d**) and after (**e**) being inactivated by exposure to methylene blue-mediated PDT. Clear regions indicated by asterisks (*) are large protein aggregates formed by intense oxidation imposed by PDT (Transmission electron microscopy images are from C. P. Sabino's personal collection)

are most damaged. In fact, the damage ratios done by HO• and $^1O_2$ are nearly equal among nucleic acids, fatty acids, proteins, and other structures [7].

So far, most scientific investigations have focused their attention on oxidative damage to DNA and membrane lipids, while relegating protein oxidation to secondary importance. Indeed, lipid peroxidation can rapidly kill cells by membrane rupture, and DNA oxidation can also kill cells or lead to mutations. Another point is that DNA and lipid oxidation are relatively easier to experimentally quantify and are much less complex to interpret. However, over the past years, some rather important reports have been indicating that protein oxidation may actually be the key process behind ROS-derived cell signaling and toxicity. Until recently it was believed that ionizing radiation kills cells by DNA oxidation due to reaction with HO• generated by water homolysis. However, using a model of radioresistant bacteria (*Deinococcus radiodurans*), Daly et al. demonstrated that the primary reason for cellular inactivation upon gamma irradiation was actually imposed by protein oxidation [9]. This publication brought important changes to the paradigm on mechanisms of cellular inactivation by ROS and added further importance to the determinants of protein

(per) oxidation. Unfortunately, the PDT scientific community is still conducting experiments to determine whether proteins are also a primary target for cell inactivation by $^1O_2$. However, if we consider theoretical simulations, we can still expect that $^1O_2$ might also inactivate cells via protein damage, i.e., in the same way that HO• does [7]. Proteins are ultimately responsible for most cellular functions and are scattered through all compartments of all cells. Regardless of taxonomy or strain, proteins are the most abundant component of cells (Figs. 5.2a–c). In cell membranes they compose from 30 to 75 % of the total membrane dry weight [10]. Bacterial cells lack intracellular compartments or organelles. Their DNA is centrally distributed within the cell and is surrounded by large amounts of protein complexes, including transcription factors and ribosomes. In Fig. 5.2, we present transmission electron microscopy images of a Gram-negative bacterial cell before (Fig. 5.2d) and after (Fig. 5.2e) inactivation by exposure to methylene blue and red light. It seems clear that the cell membrane is not disrupted by PDT, because all the intracellular contents are still contained inside the cell. However, several clear spheres (indicated by asterisks, *) appear after irradiation. Those spheres, often referred as inclusion bodies, are large protein aggregates commonly seen in cells that were exposed to oxidative stress or intense heat. Although it is difficult to quantify, these images clearly illustrate the importance of protein oxidation during PDT.

5.3 Cell Death Pathways

Eukaryotic cells, in general, present a much more complex spatial and molecular organization when compared to prokaryotes such as bacteria. Due to the electrochemical diversity found inside each organelle of eukaryotic cells, it has been possible to employ photosensitizers with molecular structures that show a distinct tendency to accumulate in certain intracellular compartments. In contrast to what can be seen in Fig. 5.2d, e, in eukaryotic cells damage may be localized to certain organelles, while the other cellular compartments remain undamaged. Hence, ROS production can be targeted to specific structures that will trigger cell death pathways which correspond to the signaling cascades arising from specific damage to different sites (Fig. 5.3). Eukaryotic cells of metazoan animals present rather elaborate signaling pathways leading to regulate either cell death or survival. Necrosis and apoptosis are the two main types of cell death subroutines that have been classically established based on morphologic and biochemical divergences. In this classic perspective, apoptosis would be a programmed and "organized" pathway of cell death, while necrosis is classified as unregulated or "accidental" death. Even though the morphological and biochemical aspects do indeed exist, whether it is a matter of programmed or accidental, subroutine is not so simple to distinguish. Here we will describe the mechanisms based on studies of the mammalian class of organisms, because the literature still lack studies with respect to reptiles, birds, amphibians, etc.

Autophagy is often mistakenly interpreted as an independent mechanism of cell death. It actually is a "last ditch" mechanism to try to rescue severely damaged cells

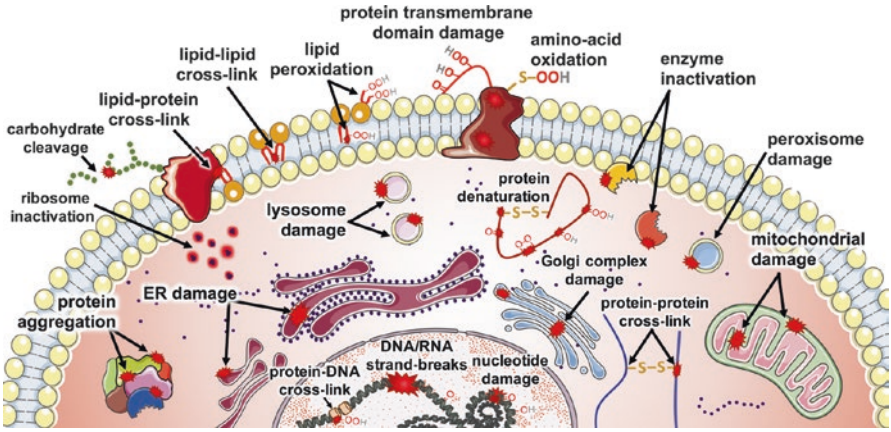

Fig. 5.3 Illustrative examples of cellular damage caused by photodynamic therapy. Note that virtually all vital structures of the cell can potentially be damaged and lead to diverse cell death pathways. As described in this chapter, the factors that determine the preferential damage site are photosensitizer localization, photodynamic reaction type, and target concentration [39]

from death. In this situation, the damaged proteins and organelles are digested in lysosomes to increase protein turnover and (by eliminating nonfunctional structures) supply amino acids to rebuild them [11, 12]. It is clear that excessive degradation of cellular structures can lead to cellular death; however, in well-controlled situations, it can indeed rescue cells from apoptosis or necrosis [13]. Autophagy can be initiated by light-activated photosensitizers that accumulate either in the cytosol, endoplasmic reticulum (ER), mitochondria, or lysosomes. After damage is detected, the autophagic signal is propagated through the cell via mTOR/AKT pathway. Interestingly, cells with a blocked apoptosis pathway either due to impaired signaling or lack of ATP may also independently initiate autophagy to promote cell death [13].

Apoptosis was initially described by pathologists from a morphological point of view. Cells undergoing apoptosis lose their original shape as they shrink and form apoptotic bodies and membrane blebs containing degraded intracellular contents. The chromatin is condensed and DNA is cleaved in regions located between nucleosomes. Apoptosis is completely regulated by genetic information, and, hence, some refer to it as "programmed cell death." Nearly all apoptotic pathways depend directly or indirectly on mitochondrial signaling via proteins from the caspase family. The intrinsic pathway can be initiated by the presence of certain ROS-damaged organelles or molecules in the cytosol that can be sensed by mitochondrial proteins. Caspase-9 signaling then promotes expression of proapoptotic genes to induce the programmed cell death routine. Alternatively, cells can undergo a caspase-independent apoptosis via release of mitochondrial proteins such as apoptosis-inducing factor (AIF), Omi/HtrA2, or endonuclease G [14, 15].

The extrinsic apoptosis pathway is originated by extracellular stimuli mediated by immune cells or activation of surface pro-death receptors. Adaptive immunity

can be directed against some particular cell types (these may be some kind of immunogenic cancer or virus-infected cells), especially if characteristic epitopes are exposed to membrane surface in a non-tolerogenic environment. PDT-mediated oxidative damage can induce a trauma in treated tissues so that damage-associated molecular patterns (DAMPs) and proinflammatory cytokines are released thereby signalizing an immunogenic environment within the damaged region [16]. Upon signaling mediated by activated antigen-presenting cells (e.g., dendritic cells and macrophages), effector T-cell ($CD8^+$) clones can be selected to proliferate with specific receptors able to recognize such cognate epitopes. The effector lymphocytes can patrol through the whole organism and locate the cells exposing their specific cognate epitope and then to induce the apoptotic death of the target cells, even in distant tumor metastasis. Extrinsic apoptosis signaling is initiated by caspase-8 and converges in the mitochondria to elicit the common apoptosis subroutine.

Necrosis has long been regarded as a severe trauma-associated cell death that is passive and uncontrolled. However, it was recently described that necrotic cell death can also be regulated by ROS, caspase-8, and receptor-interacting protein 1 (RIP1) [17]. Either way, necrosis is characterized by cellular swelling, membrane rupture, and leakage of cytosolic content including organelles, proteins, nucleic acids and other DAMPs [16, 18]. These intracellular contents are highly immunogenic and potentially lead to progression of inflammation and adaptive immunity. PDT may induce necrosis if PS accumulates in cell membrane (e.g., highly lipophilic molecules or compounds that do not enter cells) or if PDT doses are exceedingly high.

5.4 Organelle Damage

5.4.1 Ribosomes

Ribosomes are very large protein-RNA complexes that are responsible for the synthesis of all proteins within our cells. Containing both protein and RNA regions, ribosomes present several oxidation labile sites that can lead to their functional inactivation [19]. This type of organelle damage is deadly to the cell since it directly impairs protein synthesis, including translation of antioxidant defense and repair systems in response to changes in cellular redox balance.

5.4.2 Mitochondria

As mentioned above, mitochondria play a pivotal role in apoptosis signaling cascades. Many photosensitizers, especially the cationic amphiphilic ones, can selectively accumulate in mitochondria and will cause rapid permeabilization and destruction after light activation. High levels of lipid peroxidation in the external mitochondrial membrane can open the membrane permeability pores that allow

passage of caspase activators such as cytochrome c, AIF, and "second mitochondria-derived activators of caspases" (SMAC) such as DIABLO [14, 15].

5.4.3 Lysosomes

Lysosomes are organelles responsible for degradation of both intracellular and extracellular biomolecules that are damaged or unwanted. Their interior is maintained at an acidic pH level (pH 4.5–5), and they contain more than 50 enzymes responsible for the degradation of many biological substrates. Lysosome-targeted PDT can cause the membrane to rupture thereby releasing all the contents into the cytoplasm. PDT-damaged lysosomes also release cathepsins that can promote mitochondrial membrane permeabilization via cleavage of enzymes from the BH3-only family or can even directly activate caspases to initiate the intrinsic apoptosis pathway [20].

5.4.4 Endoplasmic Reticulum

Endoplasmic reticulum (ER) stress can lead to several different signaling pathways that may lead to a pro-survival outcome or to intrinsic apoptosis pathways and cell death. Which pathway will eventually decide the fate of the cell is determined by the level of oxidative stress that the organelle was exposed to. Low to mild levels of ROS can activate survival pathways via induction of autophagy, antioxidant production, and JNK and $p38^{MAPK}$ signaling pathways that are responsible for cell survival and even promotion of growth. On the other hand, exposure to high levels of oxidative stress can increase the permeability of the ER membrane causing release of calcium ions that elicit mitochondrial-mediated intrinsic apoptosis [21]. PDT-mediated ER stress has also been associated with activation of CCAAT-enhancer-binding proteins (C/EBPs) homologous protein (CHOP) that is also inducible by DNA damage. The CHOP transcripts induce expression of apoptosis-associated genes that define the cell's fate [22].

5.5 Antioxidant Defense and Resistance Mechanisms

The development of cellular antioxidant defense enzymes and pathways was a milestone in the history of evolution. About 1.5 billion years ago, the abundance of photosynthetic organisms in the planet started to impact the atmospheric O_2 concentration. At first, all life on the Earth thrived using variations of anaerobic metabolism, while O_2 represented only a toxic by-product of photosynthesis that had to be avoided to prevent cellular damage. Hence, O_2-rich environments on the Earth left gaps to be

colonized by those life-forms which could better withstand such harsh conditions. This was the turning point for natural selection of organisms that developed antioxidant defense mechanisms to keep a balanced intracellular redox status [23]. After this evolutionary turnaround, cells were able to develop new metabolic pathways to utilize the chemical energy of O_2 to facilitate ATP production via oxidative phosphorylation with much greater efficiency than the previous anaerobic fermentation process.

Proteomic studies of organisms from all the kingdoms of life currently inhabiting our planet have revealed that there is a highly conserved group of about 300 proteins that are considered to be the minimal essential set of genes that can produce a viable organism. Out of those 300 proteins, 44 (15 %) are dedicated to the response and defense against cellular stress, and 18 (6 %) are essential proteins for maintenance of the correct intracellular redox balance and avoiding oxidative damage to macromolecules and membrane lipids [4]. Therefore, the pivotal importance of cellular mechanisms of antioxidant defenses either in physiological or pathological conditions remains unquestionable. Since PDT kills cells via oxidative damage, the cell's capacity to regulate its own redox balance and prevent oxidative stress can directly influence its sensitivity to photodynamic inactivation. For this reason, PDT protocols must be developed to ensure that oxidative stress imposed to target cells surpasses their antioxidant capacity. Following, we will describe how cellular antioxidant systems work and how PDT can overcome them.

Living organisms can utilize small molecular weight antioxidants to basically act as redox balance buffers. Scavenger or quencher compounds are easily oxidized and form nonreactive products. Physical quenchers mainly dispose the excess energy of singlet oxygen by changing to a nonreactive excited state (and ground state oxygen) that rapidly decays to the ground state dissipating energy in the form of heat. So when small-molecule antioxidants are present in micromolar to millimolar intracellular concentrations, they can minimize oxidation of important biomolecules simply by competition (i.e., they interact with ROS before other molecules do it).

Some small molecular weight antioxidants are constantly produced by animal cells (e.g., urate, glutathione, melanin), and others must be acquired in the diet (e.g., vitamin C for humans, vitamin E, carotenoids). Many antioxidants obtained from diet are derived from vegetables. Plants produce large amounts of scavengers and quenchers because chlorophyll has a finite probability to undergo photodynamic side reactions that yield ROS while carrying out normal photosynthesis. This is why plant leaves that have been overexposed to the sun can become yellowish and die. After the green chlorophyll has been oxidized to colorless compounds, the yellowish color of leaves reveals the abundance of carotenoid pigments produced by plant cells to prevent oxidation of chloroplasts and other organelles. Carotenoids such as ß-carotene and lycopene are among the most potent lipophilic antioxidants. Their molecular structures characteristically present several conjugated double bounds that can rapidly scavenge radicals or singlet oxygen. Upon radical reactions, it forms a nonreactive radical where the unpaired electron is delocalized through all conjugated double bounds. Additionally, carotenoids can scavenge singlet oxygen by cycloaddition as well as physically quench it forming a nonreactive excited state. However, carotenoids are highly lipophilic and can only accumulate in membranes,

so they leave hydrophilic biomolecules relatively unprotected. This may explain why carotenoid-producing bacteria, such as *Staphylococcus aureus*, are one of the most susceptible bacterial species to photodynamic inactivation using hydrophilic photosensitizers [24].

Vitamin C (ascorbate) and E (tocopherols) are complementary small-molecule antioxidants that best perform their function when combined together. Both contain double bounds that allow efficient reaction with singlet oxygen or with radicals (and these antioxidants themselves form nonreactive radicals); however, while ascorbate is highly soluble in water, tocopherols are rather lipophilic. Tocopherols are excellent scavengers of lipid peroxyl radicals and, hence, can terminate chain reactions of lipid peroxidation. Moreover, ascorbate can then act as a reducing agent and regenerate from tocopheryl radicals. Ascorbyl radicals can then undergo dismutation reactions forming dehydroascorbate and a regenerated ascorbate or be reduced by specific enzymes (e.g., NADH-dehydroascorbate reductase family). Hence, these vitamins can be efficient antioxidants for type 1 photodynamic reactions (i.e., radical production). Tocopherol can act as a singlet oxygen quencher or a nonrecyclable scavenger, giving some degree of protection for cell membranes. On the other hand, ascorbate provides very poor cellular protection since it can only scavenge singlet oxygen at relatively low rates (see Table 5.1).

Urate (uric acid) is a product of purine degradation that accumulates in many animals that are deficient in enzymes responsible for its further decomposition to more water-soluble compounds. Birds, reptiles, Dalmatian dogs, and primates (including humans) are classic examples of urate-accumulating animals. Urate is a powerful antioxidant capable of scavenging radicals, peroxides, ozone, and singlet oxygen. Its respective oxidized radical can be recycled by ascorbate similarly to tocopheryl radicals [25]. Hence, successful PDT treatment of tumors in urate-accumulating animals may require higher doses compared to treatment of other animals although this has not yet been established in well-controlled experiments.

Reduced glutathione (GSH) is a tripeptide thiol antioxidant present in huge concentrations (1–50 mM) in nearly all eukaryotic and Gram-negative bacterial cells [3]. In eukaryotic cells, GSH is found in equal concentrations in the cytosol and nucleus, but not inside mitochondria. It is not produced in most Gram-positive bacterial species, but some species have membrane transporters that allow uptake of GSH from mammalian hosts. GSH efficiently scavenges radicals, quenches/scavenges singlet oxygen, and is an enzymatic substrate for reduction of peroxides and hydroperoxides (Fig. 5.4) [26]. In a similar manner to the cysteine residues of proteins, oxidized glutathione can form disulfide bounds with another glutathione (GSSG) or with protein thiols. Glutathione disulfides do not react with biomolecules, but can be reduced back to GSH by glutathione reductase enzymes. The cellular content of GSH is directly related to the cell's capacity to tolerate photodynamic inactivation or gamma radiation. Cells can survive longer radiation exposure times compared to their GSH-deficient counterparts. GSH provides simultaneous protection of DNA, proteins, and membrane lipids [27–29]. It has also been demonstrated that fractionated PDT doses decrease phototoxicity due to regeneration of GSH by glutathione reductases in between light doses [30].

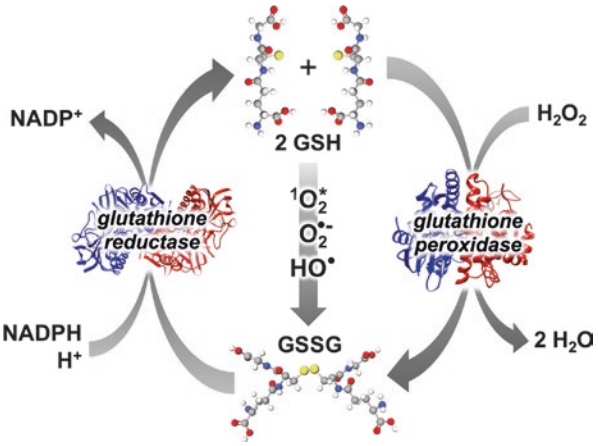

Fig. 5.4 Glutathione-dependent antioxidant defenses are broadly available in animals and microorganisms and can be highly effective to protect cells against several oxidants. The cysteine residue of reduced glutathione (GSH) can be oxidized and lead to formation of disulfide bonds with another cysteine residue of glutathione (GSSG) or proteins. GSSG can be reduced back to two GSH in a reaction catalyzed by enzymes with glutathione reductase activity, at the cost of NAPDH and H^+. Hydrogen peroxide can be reduced by two GSH molecules catalyzed by glutathione peroxidase, yielding two molecules of H_2O

In addition to small molecular weight antioxidants, cells also rely on redox-balancing enzymes that can catalyze deactivation of ROS. Among the 18 essential proteins for intracellular redox balance, the most relevant enzymes present in animal and microbial cells are superoxide dismutase (SOD), catalase (CAT), and the family of enzymes that use glutathione as a substrate. However, none of these enzymes are able to directly protect cells from singlet oxygen or hydroxyl radicals (Fig. 5.5). SOD was first described in 1969 as an abundant enzyme of vital importance present in virtually all animal tissues. SOD catalyzes the dismutation of superoxide (into hydrogen peroxide and oxygen) by several orders of magnitude, and it is indeed fundamental to protect cells from ROS generated by cell aerobic metabolism or xenobiotics. However, SOD is only able to impair photodynamic inactivation that is mediated by the few photosensitizers that preferentially undergo type 1 reactions, such as triarylmethanes (e.g., crystal or gentian violet and malachite green) and titanium dioxide semiconductors. Hence, it is best to use type 2 photosensitizers to avoid this cellular defense mechanism. The dismutation reaction produces an oxidized (O_2) and a reduced (H_2O_2) species. Therefore, superoxide dismutase detoxification must be accompanied by the decomposition of H_2O_2 to water and nontoxic products. Many enzymes such as CAT, glutathione peroxidase (GPx), and peroxiredoxin (Prx) are able to perform this task. While CAT directly catalyzes H_2O_2, dismutation to H_2O and O_2, GPx, and Prx carries out a more complex sequence of reactions. GPx catalyzes the reaction between H_2O_2 and two GSH forming H_2O and GSSG that must subsequently be regenerated to GSH by glutathione reductase. Prx has cysteine residues that can be directly oxidized by H_2O_2 forming disulfide

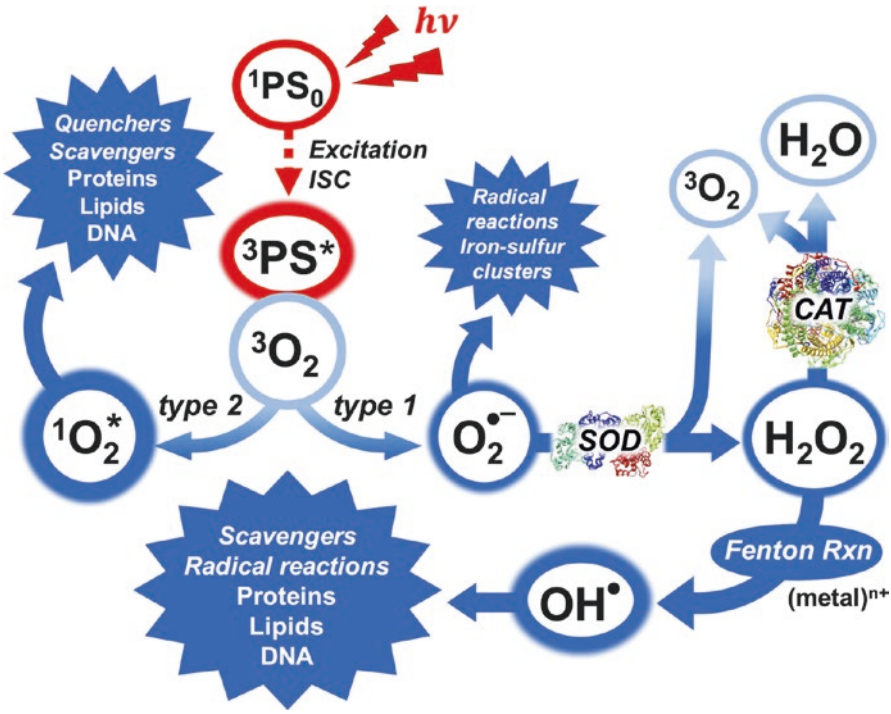

Fig. 5.5 General overview of type 1 and 2 photodynamic reactions in the cellular context. Ground state photosensitizer ($^1PS_0$) is excited by light (*hv*) to a higher energy state that decays to the long-lasting excited triplet state ($^3PS^*$) via intersystem crossing (ISC). Type 1 reaction initiates via charge transfer from $^3PS^*$ to $^3O_2$ forming superoxide radical anion ($O_2^{\bullet-}$). Superoxide can react with antioxidant scavengers, iron-sulfur clusters of enzyme active sites, or undergo dismutation reaction, catalyzed by superoxide dismutase (SOD), producing O_2 and hydrogen peroxide (H_2O_2). Hydrogen peroxide can be decomposed to H_2O and O_2 in reaction catalyzed by catalase (CAT) or other enzymes. Hydrogen peroxide can also be reduced by transition metals (e.g., Fenton reaction with Fe^{2+} or Cu^+) producing hydroxyl anions (HO^-) and radicals (HO•). Hydroxyl radicals are extremely reactive and can induce radical chain reactions in proteins, lipids, nucleic acids, and carbohydrates. Type 2 reactions produce singlet oxygen ($^1O_2^*$) via energy transfer. Singlet oxygen is also extremely reactive toward proteins, nucleic acids, and lipids. It can be quenched or scavenged by small molecular weight antioxidants, but, just like HO•, it is not impaired by any enzymes

bounds. Thioredoxin (Trx) is able to protect cells from oxidative stress by becoming oxidized itself, thus removing disulfide bounds from oxidized proteins such as Prx to restore the native protein structure. Finally, Trx needs to react with NADPH to be regenerated back to its reduced active form.

Melanin pigments constitute some of the most difficult challenges for PDT and other therapeutic modalities. In parallel to the potent antioxidant activity that makes melanotic melanomas resistant to radiotherapy and PDT, it also acts as an optical barrier for light penetration into tissues and can even sequester photosensitizer molecules due to its anionic nature. Several recent and ongoing investigations have had

some degree of success trying to overcome or, at least, minimize this limitation using tissue clearing techniques and photosensitizers that absorb infrared light (e.g., bacteriochlorins or naphthalocyanines) [31–33]. Either way, melanin-producing tumors or pathogens (e.g., *Cryptococcus neoformans*) still represent an additional challenge for nearly all commercially available photosensitizers.

Last but not least, another important resistance mechanism to PDT, also commonly expressed by drug-resistant tumors and pathogens, is the efflux of photosensitizers through membrane drug transporters [34–37]. The most commonly found types belong to the family of ATP-binding cassette transporters (ABC-transporters). In many cases the coadministration of verapamil or other efflux pump inhibitors could inhibit the resistance phenotype [35–37]. Even so, this drug is not highly specific and can cause several side effects (related to inhibition of other membrane transporters) that limit its use. Moreover, since efflux pumps are in constant contact with photosensitizer substrates, photodynamic reactions may occur in the vicinity of the pump leading to its inhibition due to oxidative damage [34]. Alternatively, other photosensitizer molecules that act in extracellular environment (e.g., large conjugated molecules or vascular targeting strategies) or that are not substrate of efflux pumps can be used to avoid resistance to PDT [34].

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