ISSN 0006-3509, Biophysics, 2016, Vol. 61, No. 3, pp. 461–469. © Pleiades Publishing, Inc., 2016.
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COMPLEX SYSTEMS BIOPHYSICS

The Biophysical Aspects of Photodynamic Therapy

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Received November 29, 2015

Abstract—The photodynamic effect, viz., photodamage of stained cells in the presence of oxygen, is used for destruction of tumors and other abnormal cells. The present review considers the biophysical mechanisms of the photodynamic action on cells. The importance of two major mechanisms of photodynamic damage of cells is discussed. The first one is mediated by electron or proton transfer, whereas the second one involves singlet oxygen. Another question that is considered is the importance of oxidation of membrane lipids or proteins for the photodynamic damage of cells. The phototransformation of photosensitizers and their intracellular localization and delivery to cells and tissues that have undergone abnormal changes are discussed. The current data on photosensitizer nanotransporters are presented. The potential sensors for reactive oxygen species in cells are discussed.

Keywords: photodynamic effect, photosensitizer, biophysics

DOI: 10.1134/S0006350916030192

The photodynamic effect is light-induced damage of cells stained by a photosensitising dye (PS) in the presence of oxygen. It is used in photodynamic therapy (PDT) for the destruction of malignant cells and microorganisms [1–3]. This multistage process includes primary photophysical processes (photon absorption and transfer of excitation energy of PSs to neighboring molecules), photochemical reactions, dark reactions, and integral cellular responses that result either in survival or death. The stages of light and photosensitizer delivery to pathologically altered tissues, tissue reactions, removal of excess dye that makes the eyes and skin photosensitive, digestion of the damaged tissue, and wound healing are also included at the organism level.

PHOTOSENSITIZERS

Photosensitizers are nontoxic dyes that well absorb light at 600–800 nm. In this spectral region, which is referred to as the therapeutic window, light is weakly absorbed by main pigments, melanin, hemoglobin, myoglobin or cellular cytochromes, and penetrates deep into the tissue [3]. The main requirements for photosensitizers include high absorption coefficients and quantum yields of singlet oxygen and other reactive oxygen species (ROS), which induce oxidative stress and cell death. Porphyrin compounds: hematoporphyrin derivatives (HpD, photofrin, photohem), chlorins (mTHPC or foscan, radachlorin, mono-L-aspartyl chlorin e_6, and talaporphin), phthalocyanines (photosens and Pc4), benzoporphyrin derivatives (visudyne or verteporfin), 5-aminolevulinic acid (ALA) and its methylated form (metvix), a natural precursor of protoporphyrin IX (PpIX), hypericin, and several others are most commonly used [1–3]. Since there is no ideal photosensitizer that satisfies all the requirements yet, chemists synthesize and test new photosensitizers.

Abbreviations used: PS, photosensitizer; PDT, photodynamic therapy; ROS, reactive oxygen species.

TWO TYPES OF PHOTODYNAMIC PROCESSES

The primary photophysical processes are illustrated by a classic Jablonski diagram [4, 5]. By absorbing a photon, a molecule is excited from the singlet ground state S_0 with anti-parallel spins of the outer electrons to the singlet excited state S_1. In 10^{-9}–10^{-8} s the molecule returns to its ground state by emitting a fluorescent photon or by nonradiative transition. Sometimes, intersystem crossing, when the spin of the outer electron is reversed, can transit a molecule into the triplet state T_1 with parallel spins of the electrons. The transition between the triplet T_1 and singlet S_0 states is forbidden; thus, its probability is small and the lifetime of the triplet state is significantly higher, 10^{-4}–10^{2} s. During this time the energy can be transferred to neighboring molecules with the formation of highly active chemical products that initiate processes that are fatal for the cell.

The main photochemical mechanisms of photodynamic processes are divided into two types. In type I reactions, photoexcited PS molecules directly react with the neighboring molecules with electron or proton transfer. As electron donors for type I photodynamic reactions serve vitamins, reduced coenzymes, flavin compounds, amino acids, unsaturated lipids, and nitrogenous bases [6, 7]. Upon electron transfer to oxygen, superoxide anion $O_2^{\bullet-}$ is formed. The probability of the direct electron transfer from the photoexcited dye to oxygen is not high, but oxygen can be reduced by semi-reduced products that are formed in type I photodynamic reactions. In cells superoxide dismutase catalyzes $O_2^{\bullet-}$ conversion into hydrogen peroxide. In the presence of iron ions the highly reactive hydroxyl radical $OH^{\bullet}$ is formed. Due to a very high redox potential ($E_0^{'} = +2.3$ V) it actively oxidizes different surrounding molecules with the formation of more complex radicals [8]. Because of the high reactivity, the $OH^{\bullet}$ lifetime in a cell is very small, the diffusion path is short, and the action is limited by the immediate vicinity to the place of the generation. However, since $OH^{\bullet}$ in photodynamic processes can be formed from the superoxide anion, which diffuses to significant distances, its effects could be rather distant from the PS molecules.

It is necessary to note that $O_2^{\bullet-}$ can serve as a messenger in the system of intracellular signaling and trigger a chain of biochemical regulatory processes, which enhance the initial signal and could lead to cell death [9, 10].

In type II photodynamic reactions the energy of photoexcitation can be directly transferred from the dye to oxygen. Usual molecular oxygen O_2 is a biradical with two unpaired electrons. Its ground state is triplet ($^3\Sigma_g^-$). It is stable because the electrons are at different oxygen atoms and the Pauli exclusion principle on the presence in one quantum system of two particles in the same quantum states is avoided. Upon energy transfer from a photoexcited PS molecule to oxygen, the latter is turned to the singlet state. Two variants of singlet oxygen are possible. In one variant, the both electrons with antiparallel spins occupy orbitals at different atoms. This form ($^1\Sigma_g^+$) is very short-lived (several picoseconds) and does not have biological significance. In the second case, both paired electrons occupy one orbital. The second orbital is free and can accept two electrons. It is this form ($^1\Delta_g$) that is referred to as singlet oxygen $^1O_2$ [11–14]. $^1O_2$ is a highly reactive electrophilic agent that is capable of oxidizing different organic substances. One of the most important targets of $^1O_2$ is unsaturated lipids of biomembranes. Hydroperoxides that are formed as a result of their oxidation initiate a chain reaction of lipid peroxidation that leads to the structural damage and disruption of the biomembranes [8, 12]. $^1O_2$ also reacts with organic molecules that contain nitrogen or sulphur, in particular, tryptophan, histidine, tyrosine, methionine, and cysteine [15, 16].

Due to the high reactivity, the $^1O_2$ lifetime in water is 2–5 μs and in cells that contain many quenchers, mainly amino acids and proteins, it is significantly smaller. According to the estimate that was made in [17], the diffusion path of $^1O_2$ in cells does not exceed 10–20 nm, and according to data of A.A. Krasnovsky it is approximately 9 nm in the cytoplasm and 4–13 nm in biomembranes [12, 13]. Therefore, $^1O_2$ can oxidize only molecules that are in the immediate vicinity of the photosensitizer. Thus, the photodynamic damage of a cell is mainly determined by the intracellular localization of the photosensitizer.

WHICH TYPE OF PHOTODYNAMIC REACTION IS MORE EFFECTIVE?

The issues of which type (I or II) of photodynamic reactions makes the greatest contribution to the photodynamic damage of cells, and what is more important, the effect on membrane lipids or proteins are debated in the literature. These questions are not simple, because the choice of the type I or II photodynamic reaction depends on many parameters, such as the PS microenvironment, ratios of reaction rates, availability of oxygen, type of targets, etc.

The radical pairs that are formed in type I reactions are more stable in aqueous solutions with a high dielectric constant, where reverse electron transport is hindered. On the other hand, the solubility and lifetime of $^1O_2$ are higher in non-polar lipid media [18, 19]. Therefore, the conditions for the type I and type II reactions are more suitable in cytosol and plasma membranes, respectively. However, due to the high reactivity of $^1O_2$, which exceeds the oxidizing capacity of normal oxygen by two orders of magnitude, the type II reactions with involvement of singlet oxygen prevail in the mechanisms of the photodynamic action of most photosensitizes (porphyrins, chlorins, phthalocyanine, etc.) [11, 14, 20].

In solutions, the photodynamic action of different PSs correlates with the quantum yield of $^1O_2$ [15, 19, 21, 22]. The results of the photodynamic effect in cells may significantly vary because the quantum yield and lifetime of $^1O_2$ depend on the localization and microenvironment of the photosensitizer molecules [18, 19]. For example, the photodynamic effects of two cationic photosensitizers, methylene blue and crystal violet, on HeLa cells were compared in [19]. Methylene blue is a typical type II PS with a high quantum yield of $^1O_2$ in different biological and chemical media. Crystal violet selectively localizes in mitochondria. The quantum

yield of the photogeneration of $^1O_2$ upon the photosensitization of cells with crystal violet is low; it acts like a type I PS. However, the photophysical effect of crystal violet appeared to be similar to that of methylene blue, although it generates a significantly smaller number of active products. In this case, the mitochondrial localization of crystal violet, but not its photochemical reactivity is crucial for its photophysical effect [19].

CHANGES IN THE MECHANISM OF PHOTODYNAMIC REACTIONS DURING PDT

The mechanism of the photodynamic effect can change during exposure. Intensive photodynamic exposure consumes oxygen and its tissue content rapidly decreases. It results in tissue hypoxia, decrease in the photogeneration of $^1O_2$, and delay in tissue damage [23–25]. This is particularly prominent in tumors, where hypoxic conditions are observed even without photodynamic treatment. Thus, to increase the photodynamic effectiveness, the radiation dose is often fractionated, and a tumor is radiated in a pulsed mode so that the blood flow has time to restore the oxygen content during pauses between light pulses [26, 27].

Good results are observed if a PS is introduced slowly with its tissue level being continuously maintained or during long-term (for several days or even weeks) irradiation of a tumor with low-intensity light. These approaches are referred to as metronomic PDT. At low-intensive exposures, cell death mainly occurs due to apoptosis, not necrosis. As an example, upon supplementing the daily ration of rats with 5-aminolevulinic acid during 10 days, the PpIX accumulation in brain tumors could be significantly increased, since ALA more easily penetrates the blood–brain barrier upon slow introduction [28]. The long-term irradiation is implemented by implantation of light-emitting diodes and optical fibers. The potential effectiveness of the metronomic approach for treatment of gliomas, brain tumors that are resistant to chemotherapy and radiation therapy, has been demonstrated [29–31].

PHOTOTRANSFORMATION OF PHOTOSENSITIZERS

Another cause of the change in the mechanism of the photodynamic effect during exposure could be photodegradation or phototransformation of the photosensitizer. PS photodegradation with the breakage of the covalent bonds and opening of the macrocycles upon intense and long-term light exposure can occur due to the direct photoexcitation of PS molecules or under the effect of singlet oxygen or radical products of the type I photodynamic reactions. The PS phototransformations can induce changes in absorption and fluorescence spectra. Photodegradation products often do not absorb visible light and do not fluoresce; therefore, this is referred to as photobleaching of the dye. For example, the exposure of hypericin solutions for 95 min to bright orange light (600 nm) induced a decrease in the light absorption and fluorescence intensity. At the same time, photoproducts that fluoresce within the band of 470–580 nm were accumulated, which was evidence of the mild phototransformation of hypericin, not photodestruction, but rather photoisomerization [32].

Upon the PS phototransformation, photoproducts that also have photosensitizing properties can be formed. For example, the photodegradation of hematoporphyrin is accompanied by a decrease in the fluorescence intensity and formation of photoproducts that absorb red light with a maximum near 640 nm and UV radiation as well. The latter indicates the opening of the porphyrin ring. The authors suggested that photoproduct 640 consists of chlorin-like molecules [33]. mTHPBS photobleaching and accumulation of a photoproduct that was identified as mTHPC, a photosensitizer that is widely applied in PDT, was observed in [34].

The photobleaching of PpIX that is formed in cells and tissues from exogenous ALA occurs only in the presence of oxygen; thus, it is the result of the direct effect of $^1O_2$. This is accompanied by the formation of a number of photoproducts with absorption maxima at 618, 655, and 670 nm, which also manifest photosensitizing properties [35, 36]. It has been suggested that the photodegradation of hematoporphyrin derivatives or PpIX occurs during epoxidation of the double bonds between rings and formation of a methine bridge, which results in the formation of stable products like bilirubin and biliverdin [37].

The photostability of photosensitizers and their photodynamic efficiency strongly depend on their intracellular localization and microenvironment. Hydrophilic PSs are usually more stable in the cytosol than hydrophobic ones. In an aqueous medium, lipophilic dyes aggregate, which prevents their application as photosensitizers. Aggregated PSs mostly lose their fluorescence and ability to generate ROS. Lipophilic and amphiphilic PS are more stable inside membranes, where they are in the monomer and not aggregate state [38].

LIPIDS OR PROTEINS?

There are different opinions on the issue of which target, proteins or membrane lipids, is more important for the photodynamic damage of cells. It is traditionally believed that the biomembrane damage plays the main role in photodynamic effect [8]. During both type I and II photodynamic reactions lipid hydroperoxides are formed in membranes. They are more polar than the initial unoxidized substances and are shifted with respect to the nonpolar lipid tails toward the polar external medium. This loosens the membrane,

increases the average area that is occupied by one lipid molecule, and could lead to phase separation of the membrane and the appearance of inhomogeneities, such as lipid rafts [39–41].

The consequences of peroxidation of the membrane lipids in photosensitized cells are not quite clear. It is thought that chain reactions of lipid peroxidation result in membrane permeabilization, leakage of ions and metabolites, suppression of metabolism, and cell lysis [8, 42, 43]. However, reaction products that lead to the biomembrane permeabilization have not been identified as yet. According to recent data, the formation of lipid hydroperoxides does not promote leakage of substances through the membranes and loss of chemical gradients. These processes rather involve shortened forms of oxidized lipids with aldehyde or carboxyl groups at their ends. These processes are connected not so much with the generation of singlet oxygen as with the direct reactions of photosensitizers in triplet states with hydroperoxide compounds and double bonds of unsaturated lipids [39, 44–46]. Lipid peroxidation products affect different signaling processes that regulate apoptosis [40, 41, 47].

Other authors believe that the protein photodamage plays a more important role [15, 16]. Singlet oxygen can oxidize SH groups in proteins, transforming them into sulfinates, sulfonates, disulfides, and thiosulfinates. Methionine is transformed into methionine S-oxide. Tyrosine and histidine form endoperoxide compounds; tryptophan is oxidized to N-formylkynurenine [16, 48]. The photo-oxidation of amino acids in proteins could occur both in the aqueous medium of the cytosol and in proteins that are embedded into membranes. Oxygen concentration is several times higher in the lipid phase of the membranes than in the aqueous medium. The membrane lipids can be oxidized both by singlet oxygen that is formed upon the photoexcitation of hydrophobic photosensitizers that are dissolved in membranes and by radical products of the type I photodynamic reaction [58]. Not only the ability of some molecules to be photo-oxidized is of importance, but also their vicinity to PS, PS ability to bind directly to various proteins or lipids, and the role of these structures in cell functioning and survival. The photodamage of cytosolic proteins differs from the damage of proteins that are embedded in biomembranes. The argument for lipids, which says that most hydrophobic PSs are accumulated in membranes and their photodynamic effectiveness correlates with lipophilicity [49, 50], in a number of cases is not of fundamental importance. Even a small number of PSs near the active center of a protein may be sufficient for its inactivation and fatal consequences for a cell. In this case, lipophilic PSs that bind better to the hydrophobic microenvironment of the active center are more effective. As an example, bengal rose at a high concentration ($n > 1$, where n is the number of PS per one molecule of the protein) nonspecifically binds to bovine serum albumin, and at low concentration ($n \ll 1$) specifically binds to the hydrophobic pocket of this protein. In the first case, the aggregation of the dye molecules results in quenching of the excited states and prevents the photodynamic generation of $^1O_2$. In the second case, $^1O_2$ is generated and the photodynamic reaction occurs according to type II [51].

There are a great number of examples where impaired functions and cell death are connected with (but not always caused by) the photoactivation of different cellular proteins. For example, methionine oxidation by singlet oxygen inactivates ion channels, calmodulin, and a number of hormones. Histidine oxidation could be responsible for the inactivation of the permeability of transition pores, while oxidation of thiol groups, on the other hand, could induce their permeability. Tryptophan and tyrosine oxidation could lead to caspase inactivation [48]. The development of necrosis of nerve cells that is induced by the photodynamic treatment is connected with inactivation of succinate dehydrogenase and disturbance of the mitochondrial structure [52]. The same picture is observed in unstained cells that are subjected to blue light due to the internal sensibilization by the endogenous flavins [53]. Another example is the connection of apoptosis that is induced by the photodynamic treatment with the photodamage of the anti-apoptotic protein, Bcl-2 [54, 55]. However, it is difficult to prove that it is direct photodynamic damage of this protein that is fatal to a cell. This effect could be mediated by a number of intermediate processes.

LOCALIZATION OF PHOTOSENSITIZERS IN CELLS

The photodynamic efficiency of photosensitizers mostly depends on their intracellular localization. Hydrophilic PSs diffuse through lipid biomembranes poorly. They are sorbed on the cellular surface and significantly sensitize the plasma membrane. They penetrate into the cell mainly due to pinocytosis and localize on the endosome and lysosome membranes. This is the path of the penetration of anionic PSs such as monoaspartyl chlorin e_6 or disulfonated aluminum phthalocyanine $AlPcS_{2a}$ [56, 57]. Positively charged porphyrins, phenothiazines, rhodamines, triarylmethanes, and cyanines are drawn into mitochondria due to their negative transmembrane potential. Their intramitochondrial concentration could exceed the cytosolic concentration by two orders of magnitude [58–60]. Lipophilic PSs are, in contrast, localized in hydrophobic regions of the plasma membrane and membranes of internal organelles. However, having reached the membrane, they cannot leave it and reach the aqueous medium. Thus, the penetration of lipophilic PSs in a cell is also hindered: they can be transported by vesicular transport rather than by free diffusion. Amphiphilic PSs with an asymmetric distribution of nonpolar and charged groups are the most

effective. They are drawn into hydrophobic regions of the membrane using their nonpolar parts and the polar groups facilitate their exit into the aqueous medium. Thus, they penetrate inside the cell [3, 15, 61–63].

Comparison of a number of cationic amphiphilic mesoporphyrins with symmetric (trans isomers) and asymmetric (cis isomers) positions of positively charged groups demonstrated that their localization in liposomes and mitochondria and the effectiveness of the photodynamic effect on red blood cells are proportional to the lipophilicity (which is assessed by the log distribution coefficient in the system of *n*-octanol and water) and inversely proportional to the number of positive charges [62]. According to data in [64], meso-tetraphenylporphyrin with asymmetric positions of positively charged groups penetrates a cell by diffusion and localizes in the mitochondria, whereas a cationic PS with symmetric positions of cationic groups penetrates by pinocytosis into the lysosomes. Further light exposure can destroy the lysosomal membrane and release the dye. This effect underlies photochemical internalization, in which toxins, proteins, or nucleic acids could be introduced inside the cell [65].

Photosensitizers that localize in mitochondria induce apoptosis especially effectively, since damage to mitochondrial membranes promotes the release of cytochrome *c* and other proapoptotic proteins [3, 66]. In the case of photosensitizers that occupy lysosomes, the photodynamic effect can induce both necrosis and apoptosis depending on the intensity of the effect and proteolytic enzymes that are released from the photodamaged organelles [67]. Photodamage of the plasma membrane, where photosensitizers are accumulated, usually results in necrosis [3].

NANOTRANSPORTERS OF PHOTOSENSITIZERS

The main problems with the photosensitization of tumors are connected with the delivery of a photosensitizer to the target tissues. Lipophilic and amphiphilic PSs that better penetrate into the cells are more effective. However, upon introduction into the blood flow they aggregate in the aqueous medium. Another problem is the target delivery of PSs to certain cells and intracellular targets. Different carriers are used to prevent aggregation of PSs, e.g., serum albumins [68] or low-density lipoproteins [69], liposomes [70], nanoparticles [71–74], and complex multiprotein aggregates [75–77]. Another approach is the synthesis of molecules with branched side groups that complicate aggregation [78].

Metallic, ceramic, or polymer nanoparticles; inorganic oxides; and quantum dots serve as nanotransporters. A great number of PS molecules can be immobilized on the surface of nanoparticles using covalent and noncovalent interactions and pores with a specific size, where the molecules are accumulated. The nanoparticle surfaces could be made with certain physical and chemical properties, hydrophilic, hydrophobic, or amphiphilic, for their delivery into certain cellular compartments, membranes, or organelles. For target delivery to certain tissues, photosensitizers or their carriers are bound to ligands that are recognized by the receptors on the surface of target cells. The nanoparticles are stable and protect the PS from enzymatic degradation or microbial attack. They can penetrate deep into the tissues through the smallest thin capillaries. They are not immunogenic. Polymer nanoparticles could be made optically transparent, not disturbing photoactivation of PSs, and biodegrading [71–74].

Natural nanoparticles are represented by serum albumins, chitosan, and hyaluronic acid. Synthetic polymers that are used are hydrophilic polyacrylamide, polyglycolide, polylactide, polycaprolactone, polyvinyl alcohol, polyethylene glycol, copolymers as poly(lactide-glycolide), and their mixtures [74]. As an example, the incorporation of hydrophobic hypericin to nanoparticles of polyvinyl alcohol made them water soluble and increased the effectiveness of their photodynamic effect on nerve cells [79]. Using polyacrylamide nanoparticles that carry contrast agents for magnetic resonance on their surface (the RGD peptide that is recognized by integrin on the endothelium surface of tumor vessels and Photofrin as a photosensitizer), it was possible to determine tumor boundary and its growth by magnetic resonance imaging. A further photodynamic effect induced necrosis of the tumor tissue [80, 81].

Inorganic nanoparticles have a core of metal oxide or metal composition and an organic shell, which stabilizes the particle and serves as a scaffold for attachment of various functional groups [74, 82]. Their advantages are their large area per unit volume and, correspondingly, a large amount of bound PS; high, almost 100%, light absorption; the possibility to impart magnetic properties for directed transport inside the organism; bright fluorescence (quantum dots); the possibility to bind drugs simultaneously; and simplicity of manufacturing. Phthalocyanine and gold nanoparticle (AuNP) conjugates demonstrated high photodynamic effectiveness [83]. Along with the photodynamic action, high absorption of light energy leads to heating and addition thermal damage of tumor tissue [73].

One example of ceramic nanoparticles that do not degrade is silica gels with organic modifications (ORMOSIL) that are coated with polyethylene glycol, polyacrylamide, etc. [72]. These nanotransporters of PSs with controlled parameters, size, shape, and porosity, are easy to synthesize; they are stable, resistant to temperature and pH fluctuations, and are not susceptible to microbial attacks. Various functional groups including monoclonal antibodies and ligands

for target delivery, are easy to immobilize on their surface. The ligands are often represented by folates, growth factors, and other biomolecules, whose receptors are located on the surface of cancer cells [73]. As an example, porous silicon nanoparticles with folates on their surface effectively delivered PpIX to cancer cells that had folate receptors. They demonstrated excellent photodynamic characteristics in vitro and in vivo [87].

Carbon nanomaterials, fullerene, nanotubes, and graphene oxides, have also been tested as nanotransporters [85–87]. Upon laser excitation (532 nm) fullerenes effectively generate singlet oxygen, superoxide anion, and hydroxyl radical [85]. However, their application is limited by complete insolubility in water. By attachment of pyrrolidine groups, it was possible to make them water soluble [85, 86]. In recent years, attention has been paid to graphene and its derivatives. Graphene oxide is cheap, well functionalized, water soluble, and stable in biological liquids. Conjugates of graphene oxide and folate, on whose surface a large number of molecules of chlorin e_6 were adsorbed due to hydrophobic interactions and π-π stacking, were synthesized in [88]. Upon interaction of these complexes with folate receptors on the cell surface they were internalized and released chlorine e_6 into the cytoplasm due to a pH change during transformation of the endosomes into the lysosomes. The photodynamic effectiveness of this complex was sufficiently higher than that of free chlorine e_6.

Quantum dots are spherical semiconductor nanoparticles with a diameter of 2–10 nm that consist of CdSe or CdS nanocrystals that are surrounded by a shell of ZnS, CdS, or ZnSe, to which a third polymer layer is applied making the surface hydrophilic and biocompatible. A photosensitizer and different functional groups for target delivery are attached to the polymer. Upon the photoexcitation of a semiconductor, resonance energy transfer from a PS molecule occurs with further generation of $^1O_2$. A part of the energy is consumed in type I photodynamic reactions with the formation of free radicals. Quantum dots absorb light within a wide spectral range; therefore, light that is outside the absorption spectrum of PS could be used for their excitation [74, 89].

An interesting new direction is the development of nanoparticles for two-photon photodynamic therapy [15, 72, 74, 90]. One of the limitations of PDT is the small depth of penetration of visible light into tissues. Infrared light penetrates significantly deeper. At high peak intensity of radiation ($>10^6$–10^8 W/cm^2) of ultrashort pico- or femtosecond laser pulses the probability of two-photon excitation of a PS, which is equivalent to the effect of visible light with a half-wavelength, significantly increases. Upon focusing the laser beam, this effect is created only in the radiation focus, whereas outside it, one-photon absorption of infrared light almost does not affect cells. As an example, the two-photon photodynamic action of the infrared radiation (780 nm) on glioma cells of glioma C6 stained with TMPyP photosensitizer encapsulated in polyacrylamide nanoparticles, was demonstrated [90]. Another example is the use of water-soluble bis-imidazolylporphyrin conjugates for the two-photon photodynamic damage of HeLa cells by femtosecond pulses of infrared light (780 nm). This induced local damage of the plasma membrane of cells that lay in the focal plane, whereas cells that were higher or lower were not damaged [91].

For target delivery of photosensitizers such as chlorine e_6 and bacteriochlorin p to the nuclei of tumor cells, modular polypeptide nanotransporters that act using biological principles of the intracellular transport were created by A.S. Sobolev and co-authors. These complexes consisted of a protein carrier (serum albumin, bacterial hemoglobin-like protein, bacterial β-galactosidase, or other polypeptide) and a ligand that is recognized by the surface receptors and stimulates receptor-mediated endocytosis (EGF, insulin, folate, etc.). This complex was combined with proteins that were capable of dissolving lysosomal membranes (diphtheria toxin or adenoviral proteins) and peptide signals of nuclear localization that promoted transport of the complexes, which were released from the damaged lysosomes, to the nucleus. These nanotransporters increased the photodynamic effectiveness of chlorins by three orders of magnitude [75–77].

CELLULAR SENSORS OF REACTIVE OXYGEN SPECIES

How do cells respond to oxidative damage and what processes lead to cellular death? The three main mechanisms of cell death are known: apoptosis, autophagy, and necrosis [92, 93]. All of them are regulated by a complex system of signal transduction. The cell chooses one of the pathways depending on the parameters of the effect (wavelength and intensity of light, type and concentration of PS), cell type, and its functional and biochemical state. A number of reviews have been devoted to description of signaling processes that regulate the responses of diverse cells to photodynamic treatment [3, 48, 94–97]; thus, they are not discussed here. We only discuss briefly the possible sensors for singlet oxygen and other ROS.

Both unsaturated lipids and some proteins are sensitive to oxidative damage. Long-lived lipid hydroperoxides (LOOH) and their short-lived radical intermediates (L$^\bullet$, LOO$^\bullet$, OLOO$^\bullet$ or OLO$^\bullet$) are nonspecific signals of oxidative damage [8]. They can move between intracellular membranes and, thus, propagate a chain of oxidative processes throughout the entire cell [98]. In this case, several unsaturated lipids, arachidonic acid, diacylglycerol, and ceramide, are second messengers that trigger cascades of signaling processes. However, there are a number of protective

mechanisms in the cells such as lipid antioxidants and enzymes that perform ROS detoxication (glutathione peroxidase, superoxide dismutase, catalase). Signaling cascades that either lead to additional expression of antioxidant proteins or to cell death upon stronger damage are triggered only if their antioxidant activity is insufficient for cell protection.

Some redox-sensitive proteins could be ROS sensors. As mentioned above, proteins with thiol groups in their active centers are especially sensitive to oxidative damage. These include tyrosine phosphatases [99], small monomeric GTPases such as Ras [100], and the mitochondrial transporter of adenine nucleotides ANT [101, 102]. Tyrosine phosphatases remove phosphate groups from activated proteins. Their active centers usually contain a highly conserved cysteine. The photoinactivation of tyrosine phosphatases prolongs the existence of phosphorylated tyrosines and, correspondingly, prolongs the activated state of phosphorylated enzymes [99]. Oxydation of cystein 118 in the active center of the Ras protein, which connects receptor tyrosine kinases with the MAP kinase cascade, affects the exchange of guanine nucleotides [100]. The ANT protein, which exchanges mitochondrial ATP by cytosolic ADP, is involved in the formation of mitochondrial permeability transition pores and following apoptosis. Photodynamic treatment induced oxidation of its thiol groups. This protein was found to be more sensitive to photodynamic treatment than other mitochondrial proteins [101–103].

Thioredoxin, a redox-sensitive disulfide reductase, also can serve as an ROS sensor. Reduced thioredoxin forms an inactive complex with ASK1 (apoptosis signal-regulating kinase-1). After oxidation, this complex is destroyed. The released ASK1 activates MKK 3, 4, 6, and 7 kinases, which phosphorylate p38 and JNK MAP kinases; the latter activate CREB, ATF-2, c-Jun, and other transcription factors. Oxidized thioredoxin activates NF-κB transcription factor, whereas reduced thioredoxin inhibits it [104]. NF-κB also could be a sensor for ROS. It is activated by singlet oxygen, which stimulates the release of its inhibitory subunit IκB [99]. Zinc fingers, a large class of transcription factors, in which a zinc ion is held by cysteine thiol and histidine imidazole, also could be a direct molecular target of singlet oxygen [48]. Recently, it has been shown that widespread TRPM2 proteins that form multifunctional Ca^{2+}-permeable cation channels are activated by ROS and could be their sensors. They mediate an increase in the intracellular Ca^{2+} level upon oxidative stress [105]. Oxidation of the simplest glutathione peptide (γ-glutamylcysteine glycine), which carries SH group, can form disulfide bridges with different proteins such as different protein kinases (PKA, PKC, and PKD), protein phosphatase, and transcription factors (NF-κB) [48].

Intracellular oxygen sensors such as HIF-1 (hypoxia-inducible transcription factor) could register a rapid decrease in the oxygen concentration in a photosensitized cell. ROS that are generated in mitochondria activate HIF-1 especially effectively. The state of the cysteine residues that are involved in the redox regulation of HIF-1 are controlled by thioredoxin. HIF-1, together with the transcription factor AP-1, controls cellular metabolism, proliferation, and survival [104, 106].

Thus, cells don't have any specific sensor for singlet oxygen and other ROS. They probably do not need it, since different cellular proteins are redox-sensitive and each of them can stimulate intracellular processes that lead to protection or death.

CONCLUSIONS AND PROSPECTS

Photodynamic therapy is a successful example of the realization of P. Ehrlich's dream about a magic bullet, a drug that finds a damaged organ and selectively affects it. PDT is already applied for the treatment of skin, lung, esophageal, stomach, and bladder cancers and age-related macular degeneration. This method has been tested for treatment of such difficult pathologies as prostate cancer, melanoma, and glioblastoma. However, for its successful application, numerous physical, technical, chemical, biochemical, cytological, physiological, and medical problems need to be solved. These include understanding the physical mechanisms of the photoexcitation of photosensitizers, energy transfer to biological substrates, interaction of light with optically inhomogeneous biological tissues, penetration of light radiation inside them, and scattering and absorption by different cellular components. The problems of dosimetry, determination of the optimal dose in each certain case, development of optimal light sources and means for delivery of light to a damaged organ, and problems of the fluorescent diagnostics of tumors should be solved. Chemists are improving photosensitizers and are attempting to make PS with the necessary physical and chemical properties. Biologists study effects of PDT on different cellular components in vitro and in vivo experiments to reveal the regulatory mechanisms that control cell responses and either lead to cell survival or death. Pharmacologists test drugs that can increase the damage to malignant cells and protect normal cells, and study the pharmacokinetics of the distribution of the photosensitizers in the body, their accumulation in different tissues, and removal after the treatment. Physicians carry out clinical trials and develop methods for clinical application of PDT.

ACKNOWLEDGMENTS

This study was supported by the Ministry of Education and Science of Russia (grant no. 790 for organization of scientific research) and the Russian Science Foundation (project no. 14-15-00068).

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Translated by E. Berezhnaya