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Review

Photosensitization and Redox Signaling

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ABSTRACT

The effect of light in combination with a chemical or a natural compound is termed photosensitization, and is known to have multiple cellular effects. Among them, modulation of gene expression is one of the most important, because it directly influences cell adaptation to novel environmental conditions. In previous years, the *cis*- and *trans*-acting genetic elements responsible for gene regulation by radiation and photosensitization, in particular, have been well characterized. The molecular mechanisms involved in the cell response revealed that an important control occurs at the transcriptional level and is coordinated by various transcription factors. The extracellular or intracellular changes mediated by photosensitization are detected by several signal transduction networks, allowing cells to mount an appropriated response in term of gene regulation. Mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinases (PI3–K) are among the most thoroughly studied of signal transduction systems and have been shown to participate in a diverse array of cellular programs. In this review, we will show how these cascades can be activated by photosensitization. A third signal type of transduction machinery, which has been shown to be activated by photosensitization, is the one leading to the activation of the Rel/NF-κB family of transcription factors. This family includes many members, most of which can form DNA-binding homo- or heterodimers. We will show that molecular mechanisms leading to NF-κB activation by photosensitization are initiated by oxidative damage. While the exact nature of the transduction intermediates is still unknown, NF-κB activation by radiation followed different pathways from those used by pro-inflammatory cytokines. Antiox. Redox Signal. 2, 301–315.

INTRODUCTION

PHOTOSENSITIZATION is a term that covers many phenomena. Generally, it refers to the action of a component (photosensitizer) of a system that causes another component of the system to react to light (generally, UV-A or visible light). The photosensitizer in its ground state serves as a chromophore (light absorber). On light absorption, the photosensitizer becomes electronically excited to a higher energy level and then can react directly with a substrate or with some other molecule, frequently oxygen, that, in turn, can react with the substrate (Fig. 1). Thus, photosensitization processes typically have an initial 'light step' followed by one or more dark steps. The photosensitizer in the dark are almost always in the singlet state, ^0S, in which the molecule has no impaired electron spins. Absorption of a photon promotes an electron to a higher molecular orbital without a change in its spin. The first excited state is also a singlet, ^1S. Few photosensitized reactions are directly mediated by this excited state because of its short lifetime (1–100 nsec). The excited state can decay to the ground state, emitting light or heat. In the case of an effective photosensitizer, there is a fast

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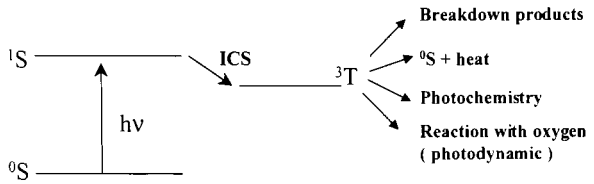

FIG. 1. The primary photoprocesses of photosensitization. ISC, Inter-crossing system.

spin inversion of the high-energy electron, resulting in a metastable triplet state, ^3T, which has two impaired electrons and a much longer lifetime (1–1,000 μ sec). The photosensitizer in its excited triplet state can undergo many collisions with other molecules during its lifetime and, as a result, can mediate photosensitized reactions with high efficiency.

In most photosensitized reactions, the photosensitizer excited in its triplet state returns to the ground state and can absorb a second photon. In a few cases, the photosensitizer is consumed in the reaction giving rise to breakdown products. Oxygen is involved in many photosensitization reactions, which are termed photodynamic. The involvement of oxygen is mediated by two pathways (Fig. 2):

1. A type I mechanism involving the photosensitizer in its excited state undergoing its primary reaction with molecules in its vicinity by an electron or hydrogen transfer process. The result is the production of a semireduced sensitizer and a semioxidized substrate. In most cases, the resultant substrate radicals react with oxygen to give rise to oxidized products of various types (*e.g.*, peroxides) that can initiate free radical autoxidation processes. The semireduced sensitizer can react with ground state oxygen to give a ground state photosensitizer and the superoxide radical anion. This latter species can also be produced with low efficiency by the transfer of an electron from the sensitizer in its excited triplet state to oxygen. Type I photosensitized reactions are most efficient at high substrate and low oxygen concentrations, because oxygen competes with substrate for interaction with triplet sensitizer to produce singlet oxygen.
2. A type II mechanism involving the triplet sensitizer interacting with ground state oxygen by energy transfer, resulting in ground state sensitizer and an electronically excited singlet oxygen state ($^1O_2$). Because both the sensitizer and ground state oxygen are triplets, their interaction does not require a change in electron spin direction and thus is efficient. The reaction of singlet oxygen with organic compounds or with biomolecules is not spin forbidden, as it is with molecular ground state oxygen. Furthermore, singlet oxygen is electrophilic, and therefore, can react with the electron-rich regions of many biomolecules to give oxidized species (hydroperoxides, endoperoxides, sulfoxides, *etc.*).

Type I

$$^3T^{\bullet-} + {}^3O_2 \longrightarrow {}^0S + O_2^{\bullet-}$$

$$^3T + {}^3O_2 \longrightarrow S^{\bullet+} + O_2^{\bullet-}$$

Type II

$$^3T + {}^3O_2 \longrightarrow {}^0S + {}^1O_2 \text{ (singlet oxygen)}$$

FIG. 2. The type I and type II photosensitization reactions.

In conclusion, photosensitization reactions can be the source of various reactive oxygen species (ROS) such as superoxide anions, hydroperoxides, hydroxyl radicals, and singlet oxygen. This propensity to generate ROS is utilized in photodynamic therapy (PDT), which is a promising new therapeutic procedure for the management of a variety of solid malignancies and is also showing interest as a modality for many nonmalignant diseases (Parrish, 1981; Pass, 1993; Godar, 1999). Typically, the treatment relies on a selective uptake of a porphyrin-based photosensitizer in a tumor relative to the surrounding normal tissue followed by light irradiation through a laser. This combination leads to oxidative damage to variety

of cellular targets and a subsequent cell death resulting in tumor ablation. For the vast majority of the photosensitizers used in PDT, singlet oxygen has been shown to be the major ROS involved in tumor eradication or in the cellular responses. Because singlet oxygen lifetime and diffusion distance in a cellular environment are limited by its avid reactivity with and quenching by cell constituents such as histidine, tryptophan, cholesterol, DNA, and RNA, it is obvious that cell damage mediated by singlet oxygen occurs close to its site of generation (Rodgers, 1985; Ryter and Tyrrell, 1998). The generation of superoxide anion by a type I reaction (Fig. 2) can also be relevant for several photosensitizers, although this ROS exhibits almost no reactivity toward biological molecules. However, it can spontaneously dismutate into hydrogen peroxide and then generate hydroxyl radicals through a Fenton-like reaction. Because the reactivity of the latter is very high with biomolecules, it instantaneously reacts at the site of its generation. On the other hand, hydrogen peroxide is a diffusible ROS due to its rather low reactivity. In the presence of transition metals, it can then undergo transformation into hydroxyl radical and then oxidize biomolecules. Hydrogen peroxide is thus the only ROS capable of generating damage to biomolecules located outside of the photosensitizer localization site.

Besides ROS generation, an important aspect of photosensitization is the cellular location of the photosensitizer itself. Five main targets have been registered:

1. All the photosensitizers, with the possible exception of the most hydrophilic ones, adsorb to the cytoplasmic membrane. In general, short-term incubation of cells with photosensitizers (*e.g.*, < 1 hr) followed by light treatment leads to more substantial damage to the plasma membrane than when the cells have been pretreated for a long time (*e.g.*, overnight) (Specht and Rodgers, 1991).
2. Endocytic uptake of photosensitizers such as protoporphyrin and hematoporphyrin derivatives or penetration of positively charged photosensitizers (acridine orange, methylene blue, *etc.*) through the plasma membrane and trapping in the acidic lysosomal compartments lead to a lysosomal or endosomal localizations. Impairment of lysosomal integrity due to lipid peroxidation is one of the main consequences of cell treatment with lysosomally located photosensitizers (Berg and Moan, 1994, 1997; Yao and Zhang, 1996).
3. Localization and damage to mitochondria by photosensitizers have been known for a long time. Among the most efficient to impair mitochondrial functions are photofrin, mesoporphyrin, chlorine 6-trimethylester, 5-aminolevulinate (ALA)-induced protoporphyrin, and benzoporphyrin derivative monoacid ring A (BPD-MA) (Kessel and Luo, 1998).
4. Several photosensitizers have been found to distribute diffusely in all intracellular membranes (endoplasmic reticulum and Golgi apparatus) (Shulok *et al.*, 1990). This includes moderately hydrophobic sensitizers which are not cationic (Woodburn *et al.*, 1991). Several pH-sensitive amphiphatic photosensitizers may penetrate lysosomal or even Golgi membranes due to the low pH of the lumen of these organelles (Malik *et al.*, 1997).
5. Many positively charged photosensitizers are located into the nucleus with several of them directly interacting with DNA through intercalation. Numerous DNA damage have been reported as consequence of photosensitization mediated by these DNA-interacting compounds (Cadet *et al.*, 1997).

Although eukaryotic cells rely on extracellular stimuli for all their functions (proliferation, differentiation, apoptosis), they also react to environmental stress from a wide variety of sources (environmental agents, oxidizing agents, radiation, photosensitization) by mounting productive responses that resemble those of growth factors. These transduction chains lead to a temporally organized activation of transcription factors and to the sequential appearance of new gene products. Since several signal transduction networks are known to respond to oxidative stress or to be influenced by ROS, it is of the utmost importance to understand how cells adapt and react to ROS in general and to oxidative stress generated by photosensitization, in particular. In this review, we

will focus on three main transduction pathways known to be influenced by photosensitization: (1) the mitogen-activated protein kinase signaling pathways, (2) the phosphoinositide kinase pathways, and (3) the NF-κB activation pathways.

THE MITOGEN-ACTIVATED PROTEIN KINASES

The members of the mitogen-activated protein kinase (MAPK) family propagate signals originating from a large collection of different external stimuli, including growth factors, hormones, cytokines, oxidative stress, and other forms of physical and chemical stress stimuli. MAPKs constitute a family of multifunctional proline-directed serine/threonine kinases activated by dual phosphorylation on both a tyrosine and a threonine residue (for reviews, see Schaeffer and Weber, 1999; Cobb, 1999; Herlaar and Brown, 1999).

MAPK cascades are evolutionarily conserved in all eukaryotes and typically organized in three-kinase architecture consisting of a MAPK, a MAPK activator (MEK [mitogen-activated/extracellular signal-regulated protein kinase], MKK [MAP kinase kinase], or MAPK kinase), and a MEK activator (MEK kinase or MAPK kinase kinase). Transmission of signals is achieved by sequential phosphorylation and activation of the components specific to a respective cascade. Four main distinguishable MAPK modules have been identified so far, but only three of them seem to be fully characterized (Fig. 3). These include the extracellular signal-regulated kinase 1 and 2 (ERK1/2) cascade, which preferentially regulates cell growth and differentiation, as well as the c-Jun N-terminal kinase (JNK)/ stress-activated protein kinase (SAPK) and p38 MAPK cascades, which function mainly in stress response like inflammation and apoptosis. Moreover, MAPK pathways control several development programs, such as morphogenesis and spatial patterning in *Dictyoselium amoebae* (Chung*et al.*, 1998), eye development in *Drosophila melanogaster* (Wassarman *et al.*, 1995), vulva induction in *Caenorhabditis elegans* (Sundaram and Han, 1996), and T-cell development in mammals (Dong *et al.*, 1998).

Because MAPK pathways form a cascade of kinases, each downstream kinase serves as a substrate for the upstream activator. It has become clear now that specificity in the transmission of the signal in the cascade is generated through direct enzyme–substrate interactions. Indeed, *in vitro* and *in vivo*, certain upstream kinases exhibit substrate specificity and preferentially signal to a subset of possible effectors. For example, members of the Raf family specifically bind to and activate MEKs (Kyriakis *et al.*, 1992), but not MKKs in the stress pathways (Minden *et al.*, 1994). The interaction of MEKs with Raf is dependent on a proline-rich sequence unique to MEKs and not found in other MKKs. Deletion of this proline-rich sequence abolishes the ability of MEK to bind to Raf and greatly diminishes the ability of Raf to activate MEK (Catling *et al.*, 1995). Likewise, MEKK1, a MAP kinase kinase of the stress pathway, appears to be specifically coupled to unique sets of activators and effectors (Yujiri *et al.*, 1998), but specificity can be lost when MEKK1 is amino-terminally truncated or overexpressed. Sequences in the carboxy-terminal domain of MAPKs that are outside of the proline-specific pocket are likely to be involved in additional enzyme–substrate interactions and may have some targeting function (Knighton *et al.*, 1991).

Various MAPKs can recognize substrates differentially, as demonstrated in a recent study that examined the interactions of ERKs, JNKs, and p38 MAPKs with transcription factor Elk-1 (Yang *et al.*, 1998). Although all three MAPK family members can phosphorylate Elk-1 *in vitro* and *in vivo*, they interact with Elk-1 through clearly distinct mechanisms. Both ERKs and JNKs associate with Elk-1 through the so-called D domain of Elk-1, but require a different set of residues within this domain. In contrast, p38 MAPK does not need the D domain for functional interaction, suggesting that this MAPK utilizes a different mechanism to select Elk-1 as a substrate. Taken together, these findings indicate that the enzyme specificity of MAPKs is determined by a combination of the intrinsic specificity of the catalytic region for serines or threonines with a proline at the +1 position, as well as domains that determine stable binding to the substrate.

The first published report on MAP kinase ac-

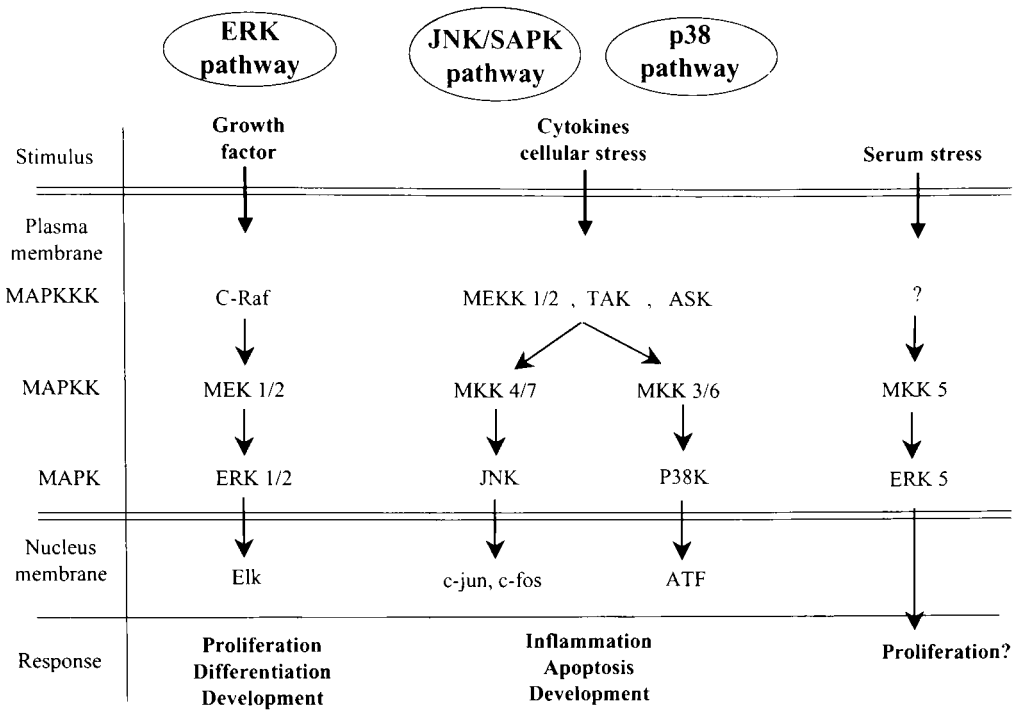

FIG. 3. A schematic overview of the mammalian MAPK modules regulating cell proliferation, differentiation, inflammation, and apoptosis.

tivation by photosensitization is by Tao *et al.* (1996). They studied the activation state of the members of the MAPK family following photodynamic treatment with BPD-MA of a naturally transformed murine keratinocyte cell line, Pam 212. The treatment causes a strong dose- and time-dependent activation of both SAPK and p38 kinase. The maximum activation of SAPK and p38 occurred between 20 and 30 min following BPD-MA treatment and 2 J/cm^2 of red light at 690 nm. In these conditions, photosensitization did not cause significant activation of ERK1/2 while under the same experimental conditions; ultraviolet light irradiation caused strong activation of SAPK/JNK and p38 with minimal activation of ERK1/2. This lack of activation of ERK1/2 is not likely due to activation of phosphatases or other negative regulatory pathways, as simultaneous treatment with BPD-MA and EGF results in an activation of ERK1/2 at a level similar to that following EGF treatment alone. A number of ROS scavengers have also been studied for their effect on BPD-MA-induced SAPK and p38 activation. Both L-histidine and *N*-acetyl-L-cysteine (NAC) showed a significant inhibitory effect on BPD-MA-induced SAPK/JNK and p38 activation, indicating that activation may be mediated by ROS. Although the mechanism of SAPK/JNK and p38 activation is still unclear, singlet oxygen or other ROS generated at the mitochondrial level where BPD-MA is located can constitute the primary triggering event.

Application of the porphyrin precursor ALA leads to the accumulation of protoporphyrin IX within mitochondria rendering cells sensitive to red light (Kennedy and Pottier, 1992). ALA-mediated PDT is efficient for neoplastic tissue because the accumulation of photosensitizing porphyrin is more pronounced in cancerous cells for several reasons, such as the faster uptake of ALA, lower activity of ferrochelatase catalyzing the formation of nonphotosensitizing heme, and an increased overall metabolic activity (Peng *et al.*, 1997). Irradiation of transformed keratinocytes preincubated with ALA for 24 hr with red light leads to a six-fold elevation of JNK and of p38 to a similar extent. Again, ERK1/2 are not activated in these conditions (Klotz *et al.*, 1998). Again, generation of singlet oxygen at the level of mitochondria could well be the triggering event of both

JNK/SAPK and p38 activation. These observations on the crucial role of singlet oxygen in MAPK activation were confirmed by the use of Rose Bengal as photosensitizer (a well-known singlet oxygen producer) and by chemiexcitation for the intracellular generation of singlet oxygen by the lipophilic 1,4-endoperoxide of *N,N'*-di (2,3–dihydroxypropyl)-1,4-naphthalenedipropionamide (Klotz *et al.*, 1999). In contrast, extracellular generation of singlet oxygen by photosensitization of Rose Bengal immobilized on agarose beads or by chemiexcitation using the hydrophilic 1,4-endoperoxide of sodium 3,3'-(1,4-naphthyldiene) dipropionate is ineffective in activation p38 and JNK (Klotz *et al.*, 1999). These observations corroborate several works showing that genes under the control of transcription factors phosphorylated by p38 or JNK such as c-*fos*, c-*jun*, metalloproteinase-1, and heme oxygenase-1, can be activated by the generation of singlet oxygen (Scharffetter-Kochanek *et al.*, 1993; Grether-Beck *et al.*, 1996; Wlaschek *et al.*, 1997). Concerning the mechanism of activation of JNK and p38 by singlet oxygen, it has been proposed that it could occur through the inactivation of a tyrosine phosphatase, especially in the case of JNK (Cavigelli *et al.*, 1995). Tyrosine phosphatases known so far rely on the presence in their active site of a cysteine that serves as a nucleophile to accept the phosphate moiety of the phosphatase substrate, forming a phosphocysteine intermediate. Oxidation of this cysteine residue would lead to the inactivation of the phosphatase, thereby allowing kinase activities to become predominant. Singlet oxygen is a well-known thiol depletor (Basu-Modak *et al.*, 1996), and it may act on MAPKs by inactivating the corresponding phosphatase. In this regard, the inactivation of membrane-bound phosphatase has been proposed to explain the net phosphorylation and activation of membrane-bound receptors targeted by UV-B and -C (Gross *et al.*, 1999; Iordanov and Magnum, 1999).

MAPK modules can affect the properties of other signaling pathways. Cross talk can affect signaling properties and information flow and can modify the specificity of the pathways involved. Elevated levels of cAMP that can induce the activation of PKA can determine which Raf isoform will be engaged in a cell to stimulate MEKs, suggesting that cAMP could function as a molecular switch to determine isoform specificity in the mitogenic MAPK cascade. Photosensitization with hematoporphyrin derivative was shown to lead to a light dose-dependent increase in intracellular cAMP levels in human bladder transitional carcinoma cells (Penning *et al.*, 1993). From this observation, it is conceivable that specificity of MAPK activation could be modulated by cAMP levels, explaining why only JNK/SAPK and p38 activation but not ERK1/2 are observed after photosensitization (Fig. 4). In addition, MAPK can affect other signaling pathways. JNK can counteract the activation of transcription factor NFAT4 by direct phosphorylation (Chow *et al.*, 1997), and can communicate with the estrogen signals by phosphorylating the estrogen receptor (Kato *et al.*, 1995). Up to now, none of this cross talk have been evidenced after photosensitization, but it is clear that an important part of the specificity of the cellular response to photosensitization originates in these interactions between transduction pathways.

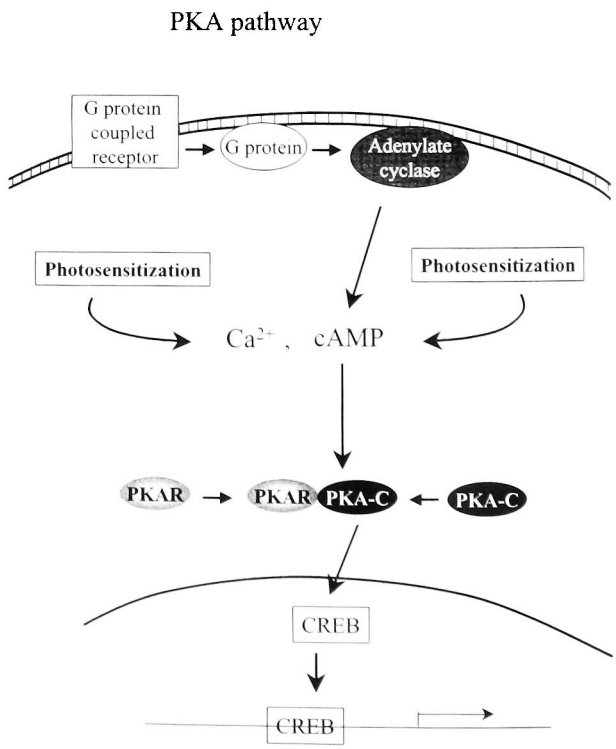

FIG. 4. The signal transduction pathway involving PKA. Photosensitization can interfere with the PKA pathway at the level of cAMP and calcium.

Increase in intracellular cyclic AMP (cAMP) levels stimulates cAMP response element binding protein- (CREB-) dependent transcription, which has also been shown to be dependent on Ca^{2+} and protein kinase A (PKA) activation (Bugrim, 1999) (Fig. 4). Taken together that the observations that photosensitization with porphyrins can trigger Ca^{2+} release from cytoplasmic (Penning *et al.*, 1992; Cui and Kanno, 1997) or mitochondrial stores (Salet *et al.*, 1997) and increase intracellular cAMP levels (Penning *et al.*, 1993), it could be postulated that CREB-dependent gene transcription be modulated by photosensitization. However, recently, cross talk between PKA and ERK has been demonstrated (Impey *et al.*, 1998). These authors showed that ERK signaling is obligatory for Ca^{2+}-stimulated transcription in PC12 cells and hippocampal neurons. The sequential activation of ERK and Rsk2 by Ca^{2+} leads to the phosphorylation and transactivation of CREB. Interestingly, the Ca^{2+}-induced nuclear translocation of ERK and Rsk2 to the nucleus requires PKA activation, explaining why PKA activity is required for Ca^{2+}-stimulated CREB-dependent transcription.

In summary, it can be mentioned that among the various MAPK cascades, two of them (JNK/SAPK and p38) have been clearly shown to be activated by several photosensitizers generating intracellular singlet oxygen. The ERK pathway is not activated under these conditions and it is still unclear whether or not the PKA pathway could be activated, although two essential co-factors (Ca^{2+} and cAMP) have been shown to be influenced by photosensitization. Actually, several genes controlled by the JNK/SAPK cascade have been shown to be transcriptionally activated by photosensitizers known to generate singlet oxygen. Among them, c-*fos* and c-*jun* are induced at least in part at the transcriptional level (Luna *et al.*, 1994; Kick *et al.*, 1996). Porphyrin-mediated photosensitization of HeLa cells results in a rapid and dose-dependent induction of both genes, but with a preferential expression of c-*jun*. Although mRNA accumulation after photosensitization was stronger and more prolonged compared with phorbol ester stimulation, with regard to AP-1 DNA-binding activity, phorbol ester was more efficient, suggesting that post-transcriptional modifications are dominant regulatory mechanisms after photosensitization, limiting the risk of deregulated oncogene expression (Kick *et al.*, 1995, 1996).

PHOSPHOINOSITIDE KINASES PATHWAY

Phosphatidylinositol, a component of eukaryotic membranes, is unique among phospholipids in that its head group can be phosphorylated at multiple free hydroxyls. Several phosphorylated derivatives, collectively termed phosphoinositides, have been involved in the regulation of diverse cellular processes such as proliferation, survival, cytoskeletal organization, vesicle trafficking, glucose transport, and platelet function. The enzymes that phosphorylate phosphatidylinositol and its derivatives are into three general families, and among them phosphoinositide 3-kinases (PI3-Ks) is the most studied (for review, see Fruman *et al.*, 1998). Among the many known signal transduction pathways, PI3-Ks have been shown to play an important role in cell survival and resistance to apoptosis (Kennedy *et al.*, 1999). Recently, the involvement of Etk/Bmx, a newly discovered tyrosine kinase that is a substrate of PI3-K, has been shown to be important in protection of prostate cancer cells from apoptosis. Parental LNCaP cells and two derivative cell lines, one overexpressing wild-type Etk (Etkwt) and the other expressing a dominant negative Etk (EtkDN), were used to study the function of Etk in apoptosis induced by photosensitization (Xue *et al.*, 1999). The cells were treated with PDT, and apoptosis was shown to be induced in LNCaP cells, as measured by DNA fragmentation and by cleavage of poly(ADP-ribose) polymerase (PARP). Moreover, the extent of apoptosis was much reduced in Etkwt cells as compared to LNCaP or EtkDN cells. Assay of overall cell viability confirmed that Etkwt cells were considerably less sensitive to PDT than were the parental LNCaP or EtkDN cells. Similar results were found in response to thapsigargin. A specific inhibitor of PI3-kinase, LY294002, abolished Etk activity and markedly increased thapsigargin (TG)-induced PARP cleavage. These results suggest

that Etk/Bmx is an efficient effector of PI3-kinase and that the newly described PI3-kinase/Etk pathway is involved in the protection of prostate carcinoma cells from apoptosis in response to PDT. The PI3-kinase pathway could also very well be protective against cell death induced to photosensitization targeting DNA because it was recently discovered that the PI3-kinase and PKB/Akt pathway delay the onset of p5-mediated apoptosis (Sabbatini and McCormick, 1999).

NF-κB SIGNALING PATHWAYS.

NF-κB and the other members of the Rel family are dimeric, sequence-specific transcription factors that control a variety of important biological events (for review, see Zandi and Karin, 1999; Karin, 1999). Unlike most transcriptional factors, proteins of this family reside in the cytoplasm and must therefore translocate into the nucleus to function. The nuclear translocation of Rel proteins is induced by an extraordinarily large number of agents such as bacterial and viral pathogens, immune and inflammatory cytokines, or a variety of agents that damage cells, such as oxidizing agents and radiation. Remarkably, an even larger number of genes appear to be targets for activation by Rel proteins.

IκB proteins form a family with a core composed of six or more ankyrin repeats, an amino-terminal regulatory domain, and a carboxy-terminal domain that contains a PEST motif (for review, see Baldwin, 1996). By binding to NF-κB dimers, the IκBs mask their nuclear localization signal (NLS), causing their cytoplasmic retention. Potent NF-κB activators can induce almost complete degradation of IκBs (especially IkBα) within minutes (for review, see Israel, 1995). This process, which is mediated by proteasome 26S (Alkalay *et al.*, 1995; DiDonato *et al.*, 1995) depends on phosphorylation of two conserved serines (Ser-32 and Ser-36 in IκBα) in the amino-terminal regulatory domain. These phosphorylations are the signal for IκBα degradation, generating active Rel dimeric complexes that translocate to the nucleus and activating genes containing Rel protein-binding sites (κB sites). Because the S32/S36AIκBα mutant is resistant to phosphorylation induced by various stimuli (interleukins, tumor necrosis factor, phorbol esters, etc.), different signaling pathways seem to result in the phosphorylation of these amino acids.

The phosphorylation event on Ser-32 and -36, which is carried out by a multisubunit kinase of high molecular weight (>700 kDa)(signalsome), is required for an additional modification of IκBα at Lys-21 and -22, namely, multiubiquitination (DiDonato *et al.*, 1997; Mercurio *et al.*, 1997). Thus, it became clear that (1) phosphorylation of IκBα preceedes ubiquitination and (2) ubiquitination is a signal for IκBα degradation. Two groups have systematically fractionated activity that phosphorylated IκBα_at Ser-32 and Ser-36 (DiDonato *et al.*, 1997; Mercurio *et al.*, 1997). The kinase activity was associated with the signalsome complex. The fraction with the highest activity was enriched with polypeptides of 85, 87, and 64 kDa. Microsequence analysis of the 85-kDa polypeptide followed by a partial cloning of the cDNA revealed that it was a previously identified Ser/Threo kinase of unknown function called CHUK. The CHUK kinase has been renamed IKK-α or IKK-1 (745 amino acids), while another related kinase found in the complex has been designated IKK-β or IKK-2 (756 amino acids). Both kinases contain a canonical MAPKK activation loop motif (SxxxS), suggesting that they are direct targets of MAPKKKs such as NIK or MEKK1 (Karin and Delhase, 1998). IKK-α and-β form homo- and heterodimers with each other, but the active form of the protein *in vivo* may be the heterodimer (Fig. 5). The formation of the heterodimers requires a leucine zipper motif (Mercurio *et al.*, 1997; Woronicz *et al.*, 1997; Zandi *et al.*, 1997). A helix-loop-helix motif might mediate interactions between the IKKs and other proteins of the IκB kinase complex and is required for efficient kinase activity (Zandi *et al.*, 1997). It has been found that IKK-α can phosphorylate the Ser-32 and -36 of IκBα efficiently, as can IKK-β (Regnier *et al.*, 1997). By contrast, although IKK-β can equally phosphorylate Ser-19 and -23 of IκB-β, IKK-α phosphorylates IκB-β poorly and appears to target Ser-23 alone. The specificity of both kinase subunits for serine has also been verified by replacing the two serine residues of IκBα and -β with either threonine or alanine. These mutations inhibited the ability of IKK-α and -β to phosphorylate the

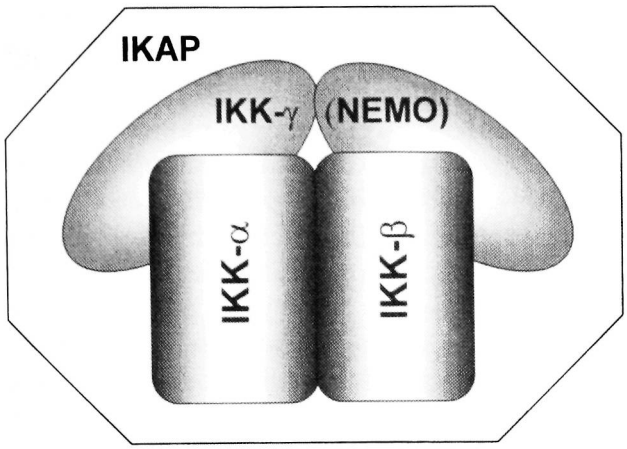

FIG. 5. Schematic representation of the IKK core complex. Two IKK-γ (NEMO) are associated with a IKK-α/IKK-β heterodimer hold together by a scaffold protein (IKAP).

IκBs, demonstrating the exquisite serine specificities of the kinases. Two candidate IKK kinases are NIK and MEKK1 (Lee *et al.*, 1998; Nakano *et al.*, 1998), but their physiological roles are not clear (Karin and Delhase, 1998). NIK preferentially phosphorylates IKK-α (Ling *et al.*, 1998), whereas MEKK1 preferentially phosphorylates IKK-β (Nakano *et al.*, 1998). Mutations within the activation or T loops of either IKK-α or IKK-β have been reported to prevent kinase activation or generate constitutively active IKKs (Mercurio *et al.*, 1997; Ling *et al.*, 1998). Recently, it has been shown that IKK activity in TNF-stimulated cells is due to the T loop phosphorylation of IKK-β, but not IKK-α (Delhase *et al.*, 1999). Once activated, IKK-β undergoes progressive autophosphorylation at multiple serines located next to its carboxy terminus. This phosphorylation decreases the kinase activity and contributes to the transient nature of IKK activation (Delhase *et al.*, 1999).

A third polypeptide of the signalsome (IKK-γ/NEMO) exhibits regulatory functions (Yamaoka *et al.*, 1998; Rothwarf *et al.*, 1998) but does not contain a recognizable catalytic domain. It is composed of three large α-helical regions, including a leucine zipper. Biochemical analysis indicates that the prediminant form of IKK is a heterodimer associated with either a trimer or dimer of NEMO (Fig. 5). An IKK complex-associated protein (IKAP) has also been described and proposed to be involved in IKK activation (Cohen *et al.*, 1998), but is not a readily detected constituent of the IKK complex; therefore, its physical significance and function are not clear.

OXIDATIVE STRESS IS IMPORTANT FOR NF-κB ACTIVATION

It has been suggested that one common step in all the activation mechanisms that lead to IκB-α degradation and NF-κB translocation involves the intracellular generation ROS (Schreck *et al.*, 1991, 1992a; for review, see Li and Karin, 1999). This conclusion was reached based upon the inhibition of NF-κB activation by a series of antioxidants. Nevertheless, the extent of this blockage appears to vary with the cell type and the nature of the signal. Inhibitory antioxidants with diverse chemical properties include NAC, dithiocarbamates, vitamin E derivatives, glutathione peroxidase activators, and various metal chelators. Support for the involvement of ROS as a common messenger also derives from evidence showing an elevated cellular level of ROS in response to tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), phorbol myristate acetate (PMA), lipopolysaccharide (LPS), and okadaic acid which all are very potent NF-κB activators (Schreck *et al.*, 1992b; Bonizzi *et al.*, 1996, Shrivastava and Aggarwal, 1999) (Fig. 6). Further evidence for an essential role of ROS came from experiments using exogenous prooxidants (Schreck *et al.*, 1991). Genetic evidence for an involvement of peroxides also came from a recent study with catalase- and Cu/Zn superoxide dismutase (SOD)-overexpressing cells. In a catalase-overexpressing cell line, NF-κB activation was substantially suppressed compared with the parental cells, whereas it was superinduced in a Cu/Zn-SOD-overexpressing cell line (Schmidt *et al.*, 1995). However, overexpression of Mn-SOD suppresses TNF-induced apoptosis and activation of NF-κB in MCF-7 cells, demonstrating that the signaling pathways activated by TNF required intramitochondrial ROS generation (Manna *et al.*, 1998). Stimulation of glutathione peroxidase (GPx) activity by selenium supplementation or GPx overexpression decreased peroxide-, IL-1β-, or TNF-α-induced NF-κB activation (Sappey *et al.*, 1994; Kretz-Remy *et al.*, 1996). Likewise, overexpression of γ-glutamylcys-

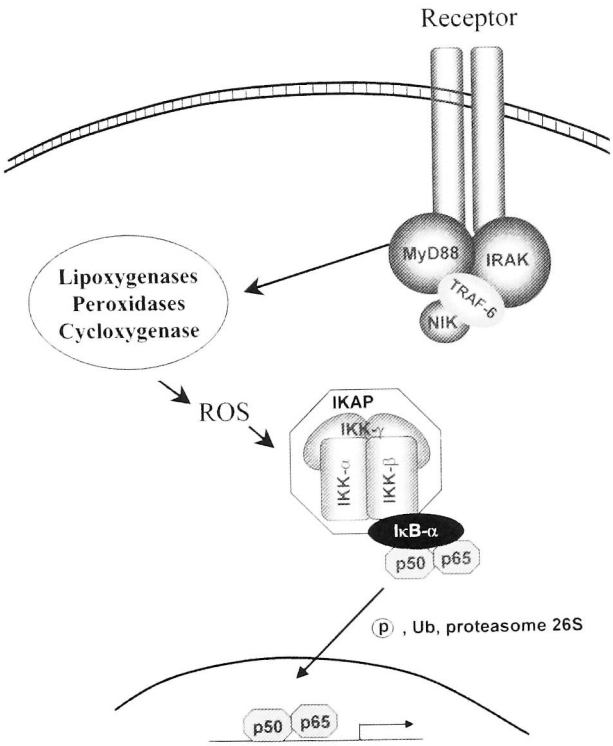

FIG. 6. ROS generated intracellularly after receptor stimulation (for example, the IL-1 receptor) feed into the NF-κB transduction pathway. Similarly, singlet oxygen ($^1O_2$) generated by photosensitization could also affect the NF-κB transduction machinery, possibly at the level of the IKKs core complex.

teine synthetase, regulating glutathione (GSH) synthesis and therefore GPx activity, suppresses TNF-α-induced activation of NF-κB in rat hepatoma cells (Manna *et al.*, 1998, 1999). These observations broaden the scope for possible oxidant involvement in NF-κB activation from peroxides to the products generated by multiple lipoxygenases and cyclooxygenases (Bonizzi *et al.*, 1997) (Fig. 6). Indeed, the classical GPx and the phospholipid hydroperoxide glutathione peroxidase have been shown, in the presence of GSH, to decrease the activity of cyclooxygenase (Warso and Lands, 1985), 5-lipoxygenase (Weitzel and Wendel, 1993), and 15–lipoxygenase (Schnurr *et al.*, 1996). Although several lines of evidence suggest a role for ROS as common and critical intermediates for various NF-κB-activating signals, the extent of their role appears to vary depending on the cell type and stimulus (for review, see Bonizzi *et al.*, 2000; Bowie *et al.*, 2000). For example, TNF-induced NF-κB is sensitive to pyrrolidine dithiocarbamte (PDTC) in a transformed human endothelial cell line, but insensitive in primary endothelial cells (Bowie *et al.*, 1997) and IL-1-induced NF-κB is not ROS-dependent in several epithelial cells mainly due to a lack in the 5-lipoxygenase-FLAP complex (Bonizzi *et al.*, 1999). From the data accumulated up to now, certain cell types, but certainly not all, rely on ROS generation for NF-κB activation. The reason for this cell selectivity is still not understood and the molecular basis for this ROS-mediated regulation is still largely unknown, but it could be postulated that a component of the signalsome could be redox-sensitive in several cell types.

Thioredoxin (TRx), which is transiently expressed or added to the culture medium, inhibits NF-κB activation after treatment with PMA (Schenk *et al.*, 1994; Hirota *et al.*, 1999). TRx is a cellular reductant for NF-κB, enabling its binding to κB sites, but it does not reduce NF-κB complexed to IκB nor does it facilitate the dissociation of the NF-κB/IκB complex (Hayashi *et al.*, 1993). Thus, it could be postulated that ROS are not counteracted in the cytosol by TRx. However, ROS must be antagonized by TRx in the nucleus to guarantee proper NF-κB binding to κB sites and gene transactivation.

The extracellular generation of singlet oxygen by the thermal decomposition of endoperoxide cannot act as a NF-κB activator, nor does it lead to transcriptional activation of a reporter gene driven by the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) (Schreck *et al.*, 1992b). To determine whether an intracellular generation of singlet oxygen would be a signal triggering NF-κB activation in T cells, these cells were subjected to photosensitization mediated by either Methylene Blue, Rose Bengal, or Proflavine. These molecules exhibit rather important singlet oxygen quantum yield and can be uptaken by cells either before or during the photosensitization reaction (Legrand-Poels *et al.*, 1993, 1995; Piret *et al.*, 1995). Electrophoretic mobility shift assay (EMSA) experiments have been performed with nuclear extracts from photosensitized cells (about 50% cell survival) showing that, in phototreated T cells, an activated factor was capable of binding to the κB sites of the HIV-1 enhancer. The intensity of the specific complex was not affected by cycloheximide, demon-

strating that the induction of this complex occurred through a post-transcriptional mechanism. Using antibodies directed against proteins of the NF-κB family, supershift experiments showed that the NF-κB factor induced by Methylene Blue, Proflavine, or Rose Bengal photosensitization is the heterodimeric form p50/RelA of NF-κB. Unexpectedly, NF-κB induction by Proflavine-mediated photosensitization was not affected by the presence of an antioxidant like NAC. All of the data obtained with photosensitizing drugs that generate singlet oxygen inside cells indicate that this ROS is capable of triggering signaling events initiated by oxidized targets or ROS, and especially that peroxides and singlet oxygen can somehow feed into the signalosome enabling IKKs to phosphorylate IκBα efficiently (Fig. 6).

Photosensitizers used in phototherapy and known to generate singlet oxygen were also shown to activate NF-κB. Photofrin-II, for example, was shown to enhance NF-κB DNA binding activity in mouse L1210 cells (Ryter and Gomer, 1993). On the other hand, pyropheophorbide derivatives, in conditions where they generate photocytotoxicity, also activate NF-κB translocation in colon cancer cells (Matroule *et al.*, 1999). In that case, the activation is clearly biphasic, with a first activation occurring within 30 min after the treatment and a second one starting 120 min after the photoreaction and sustained for at least 24 hr. The mechanisms governing such a biphasic translocation are still unknown, but it is striking to note that pyropheorbide derivatives can promote IL-1 receptor internalization and mobilize its transduction machinery. Indeed, NF-κB activation in these conditions depends on protein associated to the IL-1 receptor, such as TRAF-6, and on IKKs for IκBα degradation and subsequent RelA/p50 translocation.

Activation of NF-κB in cancer cells after PDT must have various consequences for cells receiving a nonlethal dose of light and escaping the treatment. Indeed, NF-κB can act as a transcription factor not only to turn on cytokine (IL-1, IL-2, IL-6, TNF-α) or chemokine (IL-8, MIP-1α, Rantes) gene expression (Baeuerle and Henkel, 1994) leading to an immunomodulation, but also to counteract apoptosis (Baichal and Baeuerle, 1997). Inhibition of NF-κB in cancer cells by the overexpression of the mutated form of IκBα could render cancer cells more sensitive to the apoptotic effect of PDT. Evidence of crosstalk between MAPK and NF-κB signaling pathways has recently been described. Indeed, cell stress (sodium salicylate, sorbitol, and hydrogen peroxide) can inhibit TNF-induced phosphorylation and degradation in a p38–dependent manner but have a much weaker inhibitory effect upon IL-1–induced NF-κB activation (Alpert *et al.*, 1999). This p38-dependent effect turns out to be mediated by MKK6b activation. On the other, the other MAPK pathway (ERK1/2) seems to have a positive effect on a cytokine-dependent activation of latent HIV-1, which is known to be dependent on NF-κB (Yang *et al.*, 1999). To explain this effect, the authors suggested a synergistic transactivation of the HIV-1 LTR through the NF-κB sites and a subsequent physical and functional interaction between AP-1 and NF-κB. Because MAPK pathways, especially JNK and p38, have been shown to be selectively activated by photosensitization, it would worth investigating whether activation of these cascades can inhibit cytokine signaling and NF-κB activation.

CONCLUSIONS

The way cells respond to environmental signals such as photosensitization depend on how these signals are transmitted to the cytoplasm and then to the nucleus and interpreted by cells in terms of modulation of gene expression. Three important signaling cascades (MAPK, PI-3K, and NF-κB) were shown to be selectively activated by various photosensitizers, but the integration of these different signals to mount an appropriated cellular response is still a complete mystery. The future challenge is to learn how these various pathways are coordinated and integrated to orchestrate an adaptated response to the diverse type of photosensitizers.

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ABBREVIATIONS

ALA, 5-aminolevulinate; BPD-MA, benzoporphyrin derivative monoacid ring A; cAMP, cyclic AMP; CREB, cAMP response element binding protein; EMSA, electrophoretic mobility shift-assay; ERK1/2, extracellular signal regulated kinase 1 and 2; GPx, glutathione peroxidase; GSH, glutathione; HIV-1 human immunodeficiency virus type 1; IKAP, IKK complex-associated protein; IKK, IκB kinase; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; LTR, long terminal repeat; MEK, mitogen-activated/extracellular signal-regulated protein kinase; MAPK, mitogen-activated protein kinase; MIP, macrophage-mediated inflammatory protein; MKK, MAP kinase kinase; NAC, *N*-acetyl-L-cysteine; NEMO, NF-κB essential modulator; NLS, nuclear localization signal; PARP, Poly'ADP)ribose polymerase; PDT, photodynamic therapy; PDTC, pyrrolidinedithlocarbamate; PI3-K, phosphoinositide-kinase; PKA, protein kinase A; PMA, phorbol myristate acetate; RANTES, regulated upon activation, normal T cell expressed and secreted; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; SOD, superoxide dismutase; TG, thapsigargin; TNF, tumor necrosis factor; TRx, thioredoxin.

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