Pyropheophorbide-a Methyl Ester-mediated Photosensitization Activates Transcription Factor NF-κB through the Interleukin-1 Receptor-dependent Signaling Pathway

(Received for publication, July 1, 1998, and in revised form, November 4, 1998)

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Pyropheophorbide-a methyl ester (PPME) is a second generation of photosensitizers used in photodynamic therapy. We demonstrated that PPME photosensitization activated NF-κB transcription factor in colon cancer cells. Unexpectedly, this activation occurred in two separate waves, i.e. a rapid and transient and a second slower but sustained phase. The former was due to photosensitization by PPME localized in the cytoplasmic membrane which triggered interleukin-1 receptor internalization and the transduction pathways controlled by the interleukin-1 type I receptor. Indeed, TRAF6 dominant negative mutant abolished NF-κB activation by PPME photosensitization, and TRAF2 dominant negative mutant was without any effect, and over-expression of IκB kinases increased gene transcription controlled by NF-κB. Oxidative stress was not likely involved in the activation. On the other hand, the slower and sustained wave could be the product of the release of ceramide through activation of the acidic sphingomyelinase. PMME localization within the lysosomal membrane could explain why ceramide acted as second messenger in NF-κB activation by PPME photosensitization. These data will allow a better understanding of the molecular basis of tumor eradication by photodynamic therapy, in particular the importance of the host cell response in the treatment.

Photodynamic therapy (PDT) is a new cancer treatment modality that selectively destroys malignant, premalignant, and benign lesions in patients (1–3) and is initiated by the selective accumulation of a photosensitizing agent in malignant tissue. The sensitizer is harmless unless and until activated by light of the appropriate wavelength. This results in a photochemical reaction leading to tumor destruction through a combination of direct photodamage to cancer cells as well as tumor stroma, especially the microvasculature of the tumor bed and the surrounding tissues (4). The majority of photodynamic agents is provided exogenously via intravenous injection with subsequent uptake of the drug by tissues. The only drug currently approved for therapy is a porphyrin oligomer (Photofrin) which is highly effective but exhibits several drawbacks such as (i) a tendency to cause prolonged skin photosensitivity; (ii) an activation wavelength lower than that optimal for effective penetration through tissue; and (iii) a poorly defined chemical composition that makes a detailed understanding of its mode of action and pharmacokinetics difficult. To address these issues, new photosensitizers are being developed, and a number of new agents are now in clinical trials. Several groups have recently reported the antitumor efficacy of phophorhphorbide- and pyropheophorbide-based photodynamic therapy (5–7). These compounds are chemically well characterized, absorb light above 600 nm, and produce less long term normal tissue phototoxicity than Photofrin. In the pyropheophorbide-a series, either as methyl esters or as carboxylic acids, photosensitizing efficacy increases with the length of the alkyl ether side chain, but the alkyl ether derivatives, although having similar photophysical properties (singlet oxygen and fluorescence yields), exhibit remarkable differences in photosensitizing efficiency (8). These results suggest that besides hydrophobicity, steric factors and conformation of the alkyl side chains influence localization in the cells. There are several lines of evidence suggesting that singlet oxygen (1O2) is the major damaging species in PDT (9–12). Other reactive oxygen species (ROS) may also be involved in the biological effects caused by PDT (13), particularly in the case of porphyrin derivatives used as photosensitizers (8, 14). Photochemically targeted 1O2 is mainly responsible for cytotoxicity caused by PDT. If target cells are not destroyed, photooxidative stress may modulate the activation of nuclear transcription factors that regulate the expression of stress response genes. The cellular redox state is known to be involved in regulation of gene expression, and ROS may act as chemical messengers modulating gene expression via the activation of transduction pathways (15, 16). Currently, the biological effects resulting from PDT-induced changes in gene expression and signal transduction are largely unknown. As a further step toward understanding modulation of gene expression by PDT, we report here the mechanism of NF-κB activation in colon cancer cells by pyropheophorbide-a methyl ester (PPME), a

* This work was supported in part by grants from the Belgian National Fund for Scientific Research (NFSR, Brussels, Belgium), from the Concerted Action Program (University of Liège), and from Technologie (NSHR, Brussels, Belgium). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Research fellow from the Fonds de Recherche Industrielle et Agricole (Brussels, Belgium).

§ Supported by the European Community (Biotech Program).

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¶¶¶ The abbreviations used are: PDT, photodynamic therapy; PPME, pyropheophorbide-a methyl ester; 1O2, singlet oxygen; ROS, reactive oxygen species; IL, interleukin; TNF-α, tumor necrosis factor α; IκB, IκB kinase; SMase, sphingomyelinase, FCS, fetal calf serum; DiOC6, 3,3′-dihexyloxacarbocyanine iodide; EMSA, electrophoretic mobility shift assay.

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Printed in U.S.A.

The Journal of Biological Chemistry Vol. 274, No. 5, Issue of January 29, pp. 2988–3000, 1999

This paper is available on line at http://www.jbc.org
second generation photosensitizer showing great promise in PDT. Since cytokine release during PDT may have important biological effects for surrounding cells, we decided to focus our attention on transcription factor NF-κB because it is a redox-activated transcription factor involved in the control of genes encoding several important cytokines such as interleukin (IL)-1, IL-2, and IL-6 and tumor necrosis factor (TNF)-α as well as chemokines such as IL-8, regulated on activation normal T cell expressed and secreted, and macrophage inflammatory protein-1 (see Ref. 17 for review).

NF-κB complexes bind DNA as dimers constituted from a family of proteins designated as the Rel/NF-κB family. In mammals, this family contains proteins p50, p52, p65 (RelA), RelB, and c-Rel (Rel) (18, 19). These five proteins harbor a related, but non-identical 300-amino acid long Rel homology domain that is responsible for dimerization, nuclear translocation, and specific DNA-binding. In addition, RelA, RelB, and c-Rel, but not p50 or p52, contain one or two transactivating domains. p50 and p52 derive from cytoplasmic precursors named p105 and p100, respectively. NF-κB complexes are sequestered in the cytoplasm of most resting cells by inhibitory proteins belonging to the IκB family (20–23). The members of the IκB family are IκBa, IκBβ, IκBe, p100, and p105.

Following various stimuli, including the interaction of TNF-α and IL-1β with their receptors, IκBa is first phosphorylated on serines 32 and 36, then ubiquitinated at lysines 21 and 22, and rapidly degraded by the proteasome, allowing NF-κB nuclear translocation and gene activation (24, 25). In the case of these two types of cytokines, the signal transduction pathways leading to the phosphorylation and degradation of IκB proteins have recently been clarified in HeLa and L293 cells (26–30). It is included in a 700–900-kDa complex called the activated transcription factor involved in the control of genes are controlled by NF-κB because it is a redox-responsive factor that is activated by NF-κB-inducing kinase, IKK-α, -β, and -γ (26–31). Pro-inflammatory cytokines such as TNF-α or IL-1β or the bacterial outer membrane component (lipo-lysaccharide) are potent activators of NF-κB, which mediate several of their biological activities such as stimulation of the transcription in lymphocytes through the intracellular generation of oxidative stress (32, 33). However, the assumption that a similar mechanism is effective in other cell lines has not yet been demonstrated.

In this paper, we report that PPME-mediated PDT of colon cancer cells activates NF-κB, by triggering the signaling pathway controlled by the IL-1 receptor likely without involvement of ROS. By increasing IL-1 receptor internalization, PPME photosensitization also leads to the activation of the acidic sphingomyelinase (SMase) with intracellular release of ceramide (32). This ceramide can be collected through a selected filter. In the topographic mode, fluorescence light was reflected by a mirror to the bimodal cooled CCD target (1024 × 1024 pixels), which was coupled to signal recording and processing software (Photometrics, Tucson, AZ). Images of 400 × 400 superpixels (2 × 2 binning) corresponding to a 46 × 46-μm field were recorded, and the exposure time was equal to 4 s. In the spectrotopographic mode, 150 grooves/nm grating replaced the mirror. The slit was reduced to a narrow strip and used as the entrance to the grating, delineating in the object plane a 2-μm-wide strip along the whole field from which fluorescence was collected. This slit provided a spectral resolution of about 5 nm. Fluorescence photons received by the bimodal detector produced a spectrotopographic image. Such images (335 × 335 superpixels) recorded with a 2 × 2 binning can be interpolated to recover a succession on the x direction of topographic images of a 2-μm-wide strip or a succession of spectra along the y axis (46 μm), each corresponding to an area of 2 × 0.15 μm² of the strip. Exponentially growing HCT 116 cells were grown as monolayers in custom-made glass-bottom chambers designed for use with this microspectrofluorometer.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were isolated as described by a rapid micropreparation technique derived from the large scale procedure of Dignam et al. (38) based on the use of a lysis with detergent (Nonident P-40) followed by high salt extraction of nuclei (39). Binding reactions were performed for 25 min at room temperature with 7.5 μg of total protein in 20 μl of 20 mM HEPES-KOH, pH 7.9, 75 mM NaCl, 1 mM EDTA, 5% glycerol, 0.5 mM MgCl₂, 2 μg of actinomycin-D/superoxide serum albumin, 4 μg of poly[d(I-C)] 300 μM PPME, 30 μM b-lymphoid mRNA. Labeled oligonucleotides were kindly provided by Dr. Dignam, Massachusetts General Hospital, Boston, MA. Details of these oligonucleotides are those of a previous report (38).

MATERIALS AND METHODS

Cells and Reagents—Pyrophosphoribosyl methyl ester (PPME) was from Sigma and was used without any further purification. A stock solution was made in ethanol (1 mM) and kept in the dark at −20 °C. PPME was diluted in the culture medium just before use and added to exponentially growing cells. The fluorescent probes used for cellular localization studies were purchased from Molecular Probes-Europe (Leiden, The Netherlands). Radiolabeled nucleotides were from ICN (London, United Kingdom), and [32P]-labeled IL-1β and TNF-α were from NEN Life Science Products (United Kingdom) or Amersham Pharmacia Biotech (United Kingdom). Deuterium oxide was 99.8% pure from Merck (Germany). All other chemicals were of reagent grade. Anti-IκBa monoclonal antibody was obtained from C. Dargemont (Curie Institute, Paris); anti-IκBβ was obtained from Santa Cruz Biotechnology; anti-p100 monoclonal and anti-p105 polyclonal antibodies were obtained from U. Siebels (National Institutes of Health, Bethesda).

Cell Culture—The human colon carcinoma cell line HCT-116 was grown in McCoy’s 5A medium (Life Technologies, Inc., United Kingdom) supplemented with 10% fetal calf serum (FCS, Life Technologies, Inc.). Before photosensitization with PPME, HCT-116 cells were grown for 1 week in 0% FCS. HCT-116 cells overexpressing IκBα were isolated either at seeding (26–30) or in McCoys 5A medium supplemented with 2% FCS. Cell survival was determined after 24 h using trypan blue exclusion. Photosensitization with PPME was carried out to attain 50% cell survival after 24 h.

Cellular Localization and Microspectrofluorometry—Localization experiments and intracellular fluorescence spectroscopy were carried out with a proprietary microspectrofluorometer constructed around a Leitz “Diavert” inverted microscope equipped with a heated stage which was maintained at 37 ± 1 °C (36, 37). Fluorescence excitation was performed over the whole microscope field with 405 nm light from a 100-watt super-high pressure mercury lamp (~1 watt/cm² without neutral filter). A bimodal adjustable slit in the primary image plane limited the area in the microscopic field from which the fluorescence could be collected through a selected filter. In the topographic mode, fluorescence light was reflected by a mirror to the bimodal cooled CCD target (1024 × 1024 pixels), which was coupled to signal recording and processing software (Photometrics, Tucson, AZ). Images of 400 × 400 superpixels (2 × 2 binning) corresponding to a 46 × 46-μm field were recorded, and the exposure time was equal to 4 s. In the spectrotopographic mode, 150 grooves/nm grating replaced the mirror. The slit was reduced to a narrow strip and used as the entrance to the grating, delineating in the object plane a 2-μm-wide strip along the whole field from which fluorescence was collected. This slit provided a spectral resolution of about 5 nm. Fluorescence photons received by the bimodal detector produced a spectrotopographic image. Such images (335 × 335 superpixels) recorded with a 2 × 2 binning can be interpolated in the y direction of a succession of topographic images of a 2-μm-wide strip or a succession of spectra along the y axis (46 μm), each corresponding to an area of 2 × 0.15 μm² of the strip. Exponentially growing HCT 116 cells were grown as monolayers in custom-made glass-bottom chambers designed for use with this microspectrofluorometer.
NF-κB Activation by Photodynamic Therapy

Fig. 1. A, chemical structure of PPME. B, localization of PPME in HCT-116 cells. Cells were mixed with 2 μM PPME in the dark and mounted on slides before being observed by fluorescence microscopy (λex = 515–560 nm) and under phase contrast microscopy. Arrows indicated PPME localization at the cytoplasmic membranes or in several internal compartments.

Experiments were carried out as described (40), using the same EMSA protocol as described above except for the gel concentration being 4%. The sequences of the probes (Eurogentech, Belgium) used in this work are as shown in Sequence 1.

Wild-type NF-κB probe

5’-GGTTAAAGGACTTATCATCTGT
TTGCCCTGAAAGGCAGGTCT-5’

Mutated NF-κB probe

5’-GGTTAAAGGACTTATCATCTGT
TTGCCCTGAAAGGCAGGTCT-5’

SEQUENCE 1

IκBa, IκBβ, p105, and p100 Detection—IκBa, IκBβ, p105, and p100 inhibitory subunits were detected by Western blot analysis using specific antibodies. Cytoplasmic extracts were prepared at various times after the photoactivation by cell lysis with a detergent (Nonidet P-40), pelleting the nuclei, and collecting the supernatant fraction (41). Specific antibodies. Cytoplasmic extracts were prepared at various times after the photosensitization by cell lysis with a detergent (Nonidet P-40). Pelleting the nuclei, and collecting the supernatant fraction (41).

Cytoplasmic proteins were added to a loading buffer (10 mM Tris-HCl, pH 6.8, 1% SDS, 25% glycerol, 0.1 mM β-mercaptoethanol, 0.03% bromophenol blue), boiled, and electrophoresed on a 12% polyacrylamide-SDS gel and electro-transferred to Immobilon-P membranes (Millipore, Bedford, United Kingdom). Filters were incubated with anti-IκBα (1:500 dilution), anti-p105 (1:1700 dilution), or anti-p100 (1:2000 dilution) antibodies for 60 min at room temperature, then washed in PBS, trypsinized, and counted. The cell pellet was resuspended in 70 μl of 20% (w/v) bovine serum albumin and incubated for 2 h at 37 °C in a buffer containing 250 mM sodium acetate, pH 5.0, 1 mM EDTA, and 0.2 μCi/ml [choline-methyl-14C]phosphatidylcholine. Protein samples were then added to the filter membranes, centrifuged, and counted. The pellet was resuspended in 70 μl of binding buffer and loaded on 300 μl of 20% (w/v) sucrose, 1% (w/v) bovine serum albumin. After centrifugation for 5 min at 14,000 × g, the pellet was recovered and counted. Internalization was evaluated by determining the ratio between the radioactivity measured in the cell pellet versus the total radioactivity (outside + inside cells).

Transient Transfection Assays—HCT-116 cells were grown in 6-well plates for 2 days in McCoy’s 5A medium supplemented with 10% FCS and transfected with 0.1 μg of κB-Luc reporter plasmid and various amounts of expression plasmids. The total concentration of plasmid was kept at 1 μg with pRC-CMV. Plasmids were mixed in Opti-MEM (Life Technologies, Inc., United Kingdom), added to Fugene liposomes (2 μl) (Boehringer Mannheim, Germany) for 15 min at room temperature, and loaded on cells in 2 ml of McCoy’s 5A containing 10% FCS for 24 h. Then HCT-116 were treated either with TNF-α or IL-1β for 24 h or with PPME (2 μM) for 6 h and irradiated with red light (120 s). After irradiation, cells were cultivated for 15 h and then washed twice in PBS, lysed for 15 min, and centrifuged at 15,000 × g for 4 min. Luciferase activities corrected for protein amounts (Bio-Rad protein assay) were measured in supernatants.

Ceramide Generation—For total ceramide quantification, HCT-116 cells were incubated before treatment for 24 h in McCoy’s 5A medium with 2% FCS and 2 μCi/ml of 9,10-[3H]palmitic acid (ICN, United Kingdom). After lipid extraction (43) and mild alkaline hydrolysis, the lower phase of the Folch extract containing the labeled sphingolipids was evaporated. Lipids were dissolved in chloroform/methanol (2:1, v/v), spotted, and separated on an analytical TLC. Ceramide was visualized by I2 staining, and the corresponding spots were scraped and quantified by liquid scintillation counting.

Quantification of Acidic and Neutral Sphingomyelinase Activities—Sphingomyelinase (SMase) assays were performed as described previously (44, 45). HCT-116 cells were treated with or without 150 units/ml TNF-α or with 2 μM PPME and light and then incubated for various times. To measure acidic SMase activity, 50 μg of proteins were incubated for 2 h at 37 °C in a buffer containing 250 mM sodium acetate, pH 5.0, 1 mM EDTA, and 0.2 μCi/ml [choline-methyl-14C]sphingomyelin

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Maximum intensity was about 240 counts. Fluorescence excitation wavelength was 404 nm.

E, image recorded through a 645-nm cut-off filter. Maximum intensity was about 600 counts. Right panel, mM HEPES, pH 7.4, 1 mM MgCl2, and 0.2 mCi/ml [14C]sphingomyelin (Amersham Pharmacia Biotech, United Kingdom; 20,000 Bq per assay). Released radioactive phosphocholine was extracted with chloroform/methanol (2:1, v/v) and quantified by liquid scintillation counting.

RESULTS

PPME Localizes in Membranes and in Lysosomes—Since it has been previously demonstrated that many hydrophobic derivatives of porphyrin localize at the cell membrane (46), we decided to investigate whether PPME exhibited a similar cellular distribution. Incubation of HCT-116 cells in the presence of 2 µM PPME for 17 h allowed us to detect a strong PPME fluorescence at the cytoplasmic membrane and in several internal compartments (Fig. 1B). To characterize the nature of the intracellular compartments where PPME accumulated, cells were co-incubated with PPME and various fluorescent probes. Using rhodamine 123 as a fluorescent stain of mitochondria (47), it was evident that no PPME accumulated inside the mitochondria, even after long incubation times, e.g. overnight incubation (data not shown). Cells were incubated with 1 µM PPME for 18 h and then with 2 µM neutral red for the last 30 min prior to irradiation. Under this condition, in the absence of a prolonged wash, neutral red was expected to stain mainly the endoplasmic reticulum-Golgi complex (48). This was further illustrated in Fig. 2A, cells were incubated with 1 µM PPME for 18 h and 2 µM neutral red for the last 60 min. After incubation, cells were washed twice with culture medium and left in culture medium without serum and without phenol red for measurements. Spectra were averaged from three adjacent pixels. Spectra were obtained from spectrotopographic images recorded at the onset of the irradiation (full line) and 20 s later (dashed line). The fluorescence excitation and irradiation wavelength was 405 nm. C, cells were incubated for 18 h with 0.25 µM PPME and 1 µM lucifer yellow. After incubation, cells were washed twice with culture medium and left in culture medium without serum and without phenol red for measurements. Left panel, phase contrast image; middle panel, fluorescence image recorded through a 645-nm cut-off filter. Maximum intensity was about 150 counts. Fluorescence excitation wavelength was 405 nm. D, cells were incubated for 18 h with 0.25 µM PPME and 4 µM BodipyCeram. After incubation, cells were washed twice with culture medium and left in culture medium without serum and without phenol red for measurements. Left panel, phase contrast image; middle panel, fluorescence image recorded through a 645-nm cut-off filter. Maximum intensity was about 954 counts. Right panel, fluorescence recorded through a broad filter (536 nm). Maximum intensity was about 240 counts. Fluorescence excitation wavelength was 404 nm. E, cells were incubated for 18 h with 0.25 µM PPME and carbocyanine (DiOC, 2 mg/ml). After incubation, cells were washed twice with culture medium and left in culture medium without serum and without phenol red for measurements. Left panel, phase contrast image; middle panel, fluorescence image recorded through a 645-nm cut-off filter. Maximum intensity was about 1252 counts. Right panel, fluorescence recorded through a broad filter (536 nm). Maximum intensity was about 6567 counts. Fluorescence excitation wavelength was 404 nm.
with a maximum toward 570 nm was observed. Such a maximum was in agreement with neutral red localization in the endoplasmic reticulum-Golgi complex (48). It was therefore suggested that neutral red as well as some PPME co-localized in this environment, leading to the quenching of neutral fluorescence probably by singlet-singlet energy transfer from neutral red to PPME. Irreversible PPME photobleaching inhibited the energy transfer process and restored neutral red fluorescence. Cells were also incubated with 0.25 μM PPME and 1 μM lucifer yellow for 18 h. Under these conditions and as expected, the lysosomotropic dye lucifer yellow accumulated into lysosomes as shown in Fig. 2C. This figure also demonstrated that, in addition to weak uniform fluorescence, spots that perfectly match those obtained with lucifer yellow fluorescence were observed. Moreover, sequences of images obtained with a green filter to isolate the lucifer yellow fluorescence from that of PPME showed a rapid decrease of that fluorescence, thereby suggesting lysosome destabilization since no loss occurred when cells were loaded with lucifer yellow alone (data not shown). In order to confirm whether PPME could localize in the endoplasmic reticulum and/or in the Golgi apparatus, we incubated HCT-116 cells with 0.5 μM PPME during 15 h and then with BCP (4 μM) during 15 min. BCP emits a red fluorescence only in an aggregated form which is promoted in the Golgi apparatus and in the Golgi vesicles, whereas in the monomeric form it binds to internal membranes or to the cytoplasmic membrane where it emits a green fluorescence (49). As shown in Fig. 2D, BCP emitted only a green fluorescence due to a
Similarly to what was observed by EMSA (see Fig. 3), induction of IκBα was replenished after 1 h before starting again to decrease (Fig. 2C). After 24 h almost all the IκBα pool was degraded, showing that in HCT-116 cells most of the IκBα activation mechanism was controlled by IκBα degradation.

NF-κB Activation Occurs in Two Separate Waves—Determining whether photodynamic photosensitizers such as PPME which localized in cytoplasmic and in internal membranes could activate NF-κB was of interest because it could lead to important information on a possible immunomodulation by tumor cells treated by such a photosensitive drug. To this end, HCT-116 cells were incubated for 17 h in the dark with 2 μM PPME and then irradiated with red light. Nuclear extracts were prepared at various times after photosensitization and analyzed by EMSA. As shown in Fig. 3A, an important retarded band appeared transiently after irradiation with its maximal intensity observed between 10 and 30 min. The intensity of the band decreased, almost disappearing after 1 h. A second wave of NF-κB activation could then be observed 2 h after irradiation (Fig. 3A). Contrary to the first wave of activation, the second appeared slowly and was sustained up to 24 h. This is the first demonstration that an inducing agent could activate NF-κB in two separate waves as follows: a rapid and transient phase followed by a slower and sustained one. Competition experiments carried out with a wild-type or a mutated unlabeled NF-κB probe demonstrated that the upper retarded band was specific, whereas the lower one was not (Fig. 3B). To determine whether the second phase of activation was due to a post-transcriptional mechanism, HCT-116 cells were preincubated with cycloheximide for 60 min before being photosensitized by PPME. As shown in Fig. 3A, the intensity of the NF-κB band was not decreased by cycloheximide, demonstrating that the two waves of NF-κB activation were due to a post-transcriptional activation mechanism. Using antibodies directed against the various members of the Rel/NF-κB family (p50, RelA, c-Rel, RelB, and p52), we also observed that the retarded complex involved the classical p50/RelA heterodimer (Fig. 3C).

In order to ascertain whether the NF-κB complex induced by PPME and light was transcriptionally active, we transiently transfected HCT-116 cells with a κB-Luc reporter plasmid construct. As shown in Fig. 3D, transfected HCT-116 cells incubated with PPME, in the dark did not give rise to detectable κB-driven transcriptional activity, whereas photosensitization of the transfected HCT-116 cells allowed us to detect increased transcriptional activity (4-fold), demonstrating that the p50/RelA heterodimer found in cell nuclei by EMSA was transcriptionally active. On the other hand, classical NF-κB inducers such as TNF-α or IL-1β led to a 5- and 10-fold increase in the κB-driven transcriptional activity, respectively (Fig. 3D).

Western blot analysis of cytoplasmic extracts from HCT-116 cells photosensitized by PPME also showed a rapid and transient decrease in the amount of the IκBα molecule after 30 min (Fig. 4A). Analysis of the band intensity revealed that about 50% of the cytoplasmic IκBα pool was degraded after 30 min. Similarly to what was observed by EMSA (see Fig. 3A), the IκBα degradation is transient and the cytoplasmic IκBα pool was replenished after 1 h before starting again to decrease (Fig. 4A). Concomitantly to what was observed by EMSA, this second wave of IκBα degradation was slow and sustained. After 24 h almost all the IκBα pool was degraded. These data unambiguously demonstrated that NF-κB activation by PPME and red light involved IκBα degradation in two different waves, likely involving different mechanisms. The fate of three other inhibitory Rel proteins was also followed by Western blots. As shown in Fig. 4, B–D, neither IκBβ, p105, nor p100 inhibitors were degraded following PPME photosensitization, showing that in HCT-116 cells most of the NF-κB activation mechanism was controlled by IκBα degradation.

Singlet oxygen has frequently been involved as secondary messenger in cells treated by photodynamic therapy (9–12). In order to evaluate the role of this reactive oxygen species in NF-κB activation, HCT-116 cells were irradiated in PBS where H₂O was replaced by D₂O, since singlet oxygen lifetime is increased by this isotopic substitution (51). EMSA analysis...
revealed that isotopic substitution significantly increased the intensity of the p50-RelA complex found in the nucleus of HCT-116 cells photosensitized by PPME (Fig. 5A). Interestingly, this increase in band intensity was mainly detectable during the first rapid and transient phase (Fig. 5A). However, these data by themselves could not directly implicate singlet oxygen as a mediator of NF-κB activation because isotopic replacement could modify other physicochemical parameters such as excited state lifetime (51). In order to clarify this point, we decided to investigate the effects of several antioxidant molecules (e.g., singlet oxygen quenchers) on NF-κB activation by PPME photosensitization. Many studies have shown that antioxidants blocked NF-κB activation by ROS such as hydrogen peroxide (14) but also when pro-inflammatory cytokines were used as stimuli (15). The chosen antioxidants were as follows: (i) classical hydrophilic molecules such as N-acetyl-L-cysteine (40 and 50 mM added 60 min before irradiation), pyrrolidine 9-dithiocarbamate (100–500 μM added 60 min before irradiation), and Trolox (a water-soluble derivative of vitamin E, 500 μM added 90 min before irradiation); and (ii) lipophilic antioxidants such as vitamin E (100 and 400 μM added either 60 min or 24 h before irradiation) and vitamin E acetate (400 μM added 24 h before irradiation). As shown in Fig. 5B, vitamin E acetate and all other of these antioxidant molecules did not exhibit inhibitory effects on NF-κB activation in HCT-116 cells by PPME photosensitization indicating that the intracellular release of ROS was probably not involved in NF-κB activation. To determine further whether ROS were involved or not in NF-κB activation by PPME photosensitization, lipoperoxides were measured by TBARs assay. Under experimental conditions leading to maximal NF-κB activation, there was no lipoperoxide detectable in treated HCT-116 cells, confirming that NF-κB activation by PPME photosensitization did not involve ROS generation and membrane peroxidation. 

PPME Photosensitization Promotes IL-1 Receptor Internalization—Detection in HCT-116 cells photosensitized by PPME of a rapid and transient activation of NF-κB could mimic what was observed in many cell types after treatment with cytokines. To determine whether PPME photosensitization mimicked cell activation by cytokines, we investigated the effects of PPME photosensitization on cytokine receptor in terms of receptor internalization. We first looked at IL-1 receptor internalization. As shown in Fig. 6A, addition of radiolabeled IL-1β to HCT-116 allowed us to observe its internalization within 30 min of incubation at 37 °C (lanes 1–3). This internalization could be competed out by preincubation of cells with unlabeled
NF-κB Activation by Photodynamic Therapy

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IL-1 Signaling Mediates NF-κB Activation by PPME Photosensitization—The demonstration that PPME photosensitization could favor IL-1 receptor internalization yielding a rapid and transient activation of NF-κB prompted us to determine whether PPME photosensitization activates NF-κB through the IL-1 signaling pathway. To investigate this, HCT-116 cells were transfected with a κB-Luc reporter plasmid construct together with various plasmids expressing mutant or wild-type TRAF6 protein, which is associated with the IL-1 receptor (34). HCT-116 cells were then either photosensitized with PPME or treated with IL-1β or TNF-α. As shown in Fig. 7A, the expression of TRAF6 in HCT-116 cells prior to stimulation did not significantly modify κB-driven transcriptional activity in cells photosensitized with PPME or treated with IL-1β. In cells treated with TNF-α, TRAF6 overexpression slightly inhibited κB-driven transcriptional activity (Fig. 7A). However, when HCT-116 cells were co-transfected with a dominant negative version of TRAF6 (ΔTRAF6), there was a clear down-regulation of NF-κB transactivation in cells treated with IL-1β or photosensitized by PPME, i.e., luciferase activities were lower than 50% of the control with 1 μg of ΔTRAF6 (Fig. 7A). In the case of HCT-116 treated with TNF-α, co-transfection with ΔTRAF6 led also to a dose-dependent decrease in transcriptional activity, but luciferase activity remained close to the control value at the lowest ΔTRAF6 concentrations (0.1 and 0.25 μg). These data showed that PPME photosensitization and IL-1β stimulation induced similar effects on transcription controlled by a κB gene promoter, whereas TNF-α induced κB-dependent transcription through a distinct pathway.

The role of TRAF2, an adaptor molecule associated with the TNF receptor, was also investigated in the same assay (35). As shown in Fig. 7B, expression of a TRAF2 dominant negative protein (ΔTRAF2) decreased κB-driven transcriptional activity in HCT-116 cells treated with TNF-α in a dose-dependent manner, whereas there was no effect on cells photosensitized by PPME or treated by IL-1β. These data were in agreement with the experiments where the receptor internalization was measured and confirmed that PPME photosensitization could not recruit members of the TNF-signaling pathway.

Recently, IκB kinases (IKK-α and -β) have been identified as the main cellular kinases carrying out IκB phosphorylation on serine residues 32 and 36 after cell induction with pro-inflammatory cytokines (IL-1β and TNF-α) (26–29). In order to verify whether IKKs could be activated by PPME photosensitization, HCT-116 cells were co-transfected by the κB-luciferase reporter plasmid and a plasmid expressing either IKK-α or IKK-β or IKK-α and -β. As shown in Fig. 8A, overexpression of IKK-α or -β in unstimulated HCT-116 cells increased κB-driven transcriptional activity. Co-overexpression of IKK-α and -β yielded a synergistic activation giving rise to 11-fold induction of the κB-driven transcription (Fig. 8A). Similarly, overexpression of IKK-α or -β or IKK-α plus -β increased in a dose-dependent fashion κB-driven transcriptional activity in HCT-116 cells treated with either TNF-α (Fig. 8B) or IL-1β (data not shown). Similarly, when HCT-116 cells were transfected to overexpress IKKs before being photosensitized with PPME, there was a significant increase of the κB-driven transcription; this effect was particularly significant when both IKK-α and -β...
were co-expressed (Fig. 8C). To confirm the involvement of IKKs in the PPME-mediated NF-κB activation, one HCT-116 cell line overexpressing IκBα mutated at serines 32 and 36 (S32A,S36A) was constructed (MUT4). In addition, an HCT-116 cell line overexpressing IκBα mutated at tyrosine 42 (Y42F) was also generated (Tyr-42). These two cell lines were then photosensitized as described above, and nuclear extracts were prepared after various times to be analyzed by EMSA. As shown in Fig. 8D, a classical NF-κB complex can be visualized in wild-type HCT-116 cells and in HCT-116 Tyr-42 cells. On the other hand, in the cell line (MUT4) overexpressing IκBα S32A,S36A, there was no NF-κB complex induced by photosensitization demonstrating again that NF-κB activation by PPME required IKKs and IκBα phosphorylation on serines 32 and 36. These data led to identifying IKKs as the main IκBα kinases stimulated by PPME photosensitization.

**NF-κB Activation by Photodynamic Therapy**

NF-κB Activation by PPME Involves Ceramide Generation—

Ceramide is a second messenger involved in signaling pathways following TNF-α or IL-1β stimulation (44, 52). Ceramide production after cellular stimulation with pro-inflammatory
cytokines results from sphingomyelin hydrolysis catalyzed by either the acidic or the neutral sphingomyelinase (SMase). It was reported that these two enzymes are linked to distinct pathways following interaction of TNF-α with TNF receptor 1 (53) or IL-1β to its type 1 receptor (52). Since PPME photosensitization utilized IL-1 signaling proteins to activate NF-κB, ceramide production was measured at various times after photosensitization. As shown in Fig. 9A, total ceramide significantly increased as early as 10 and 30 min after photosensitization, reaching its maximal value after 2 h before gradually declining at longer times. A similar behavior was recorded in HCT-116 cells treated with TNF-α (Fig. 9A). These data demonstrated that ceramide generation occurred soon after photosensitization but remained elevated for at least 6 hours, indicating that ceramide could be considered as being a messenger of the second wave of NF-κB induction by PPME photosensitization.

Since ceramide release could be attributed to either acidic or neutral SMase activation, these two enzymatic activities were determined in HCT-116 cells treated with TNF-α or photosensitized by PPME. From the data presented in Fig. 9A, it is evident that both TNF-α and PPME photosensitization led to acidic SMase activation. This activation was transient and appeared somewhat earlier than the release of ceramide. On the other hand, neutral SMase activity was measured on extracts from HCT-116 cells treated with 150 units/ml TNF-α or photosensitized with PPME. Although the basal activity of neutral SMase was rather low in HCT-116 cells, it was increased 1.3-fold after 15 min of treatment with TNF-α (data not shown). However, no neutral SMase activation could be detected after PPME photosensitization (data not shown), demonstrating that ceramide generation following photosensitization was totally due to acidic SMase activation.

To demonstrate further the involvement of acidic SMase and ceramide in NF-κB activation by PPME photosensitization, two experiments were carried out. First, chloroquine (100 μM), an acidic SMase inhibitor, was added 60 min prior to HCT-116 cell photosensitization with PPME, and NF-κB activation was evaluated by EMSA both 30 min and 24 h after photosensitization. As shown in Fig. 10A, chloroquine addition significantly de-
increased NF-κB activation during the first (30 min) and second activation wave (24 h), showing that acidic SMase could be considered as a mediator of the response to PPME photosensitization. Second, C2-ceramide was also added to HCT-116 cells to mimic their release by the activation of acidic SMase. As shown in Fig. 10B, NF-κB activation could be clearly visualized by addition of C2-ceramide on HCT-116 cells. NF-κB activation was clearly observed both at a short and long time after stress, emphasizing the role of ceramide as second messenger in PPME photosensitization.

**DISCUSSION**

In this paper, we have shown that pyropheophorbide-a methyl ester, a second generation photosensitizer, is a strong activator of the NF-κB transcription factor. The underlying mechanism is rather uncommon; PPME localizes in membranes and promotes IL-1 receptor internalization upon photosensitization, triggering the transduction machinery linked to the IL-1 receptor. This NF-κB activation is transient and does not require oxidative stress. After 2 h, a second wave of NF-κB action gradually appeared lasting up to 24 h. During these two phases, ceramides were generated, suggesting that these lipids act as second messengers to activate NF-κB at longer times. We postulate that PPME located in the lysosomal membrane is responsible for ceramide generation, since acidic SMase, a lysosomal enzyme, is activated by the photosensitizing action of PPME.

Investigating how pyropheophorbide-a can modulate gene expression is of great importance, because tumor eradication by PDT will likely depend not only on an efficient tumor cell killing but also on the adaptation of tumor cells surviving treatment. Since many important genes involved in the control of the immune system and in the inflammatory reaction are controlled by NF-κB, we have decided to pay attention to the mechanisms by which PPME photosensitization activates NF-κB. Photosensitization has already been shown to induce NF-κB in T lymphocytes (41, 54) and in other cell types (55). Although the mechanisms have not yet been clarified, oxidative stress mediated by photosensitization is likely implicated since antioxidants inhibit NF-κB activation or cellular oxidation products can be detected. \(^{1}\text{O}_2\) is known to be the main ROS produced by photosensitization (56) and is proposed by several authors (57–59) as a second messenger in gene activation in human skin fibroblasts irradiated by UV-A. Because its lifetime can be significantly increased by deuterium substitution, the observation that NF-κB translocation is greater in a medium where H\(_2\)O is substituted by D\(_2\)O suggests that \(^{1}\text{O}_2\) could
NF-κB Activation by Photodynamic Therapy

Fig. 10. A, effect of chloroquine (100 μM) on NF-κB activation in HCT-116 cells photosensitized by PPME. Chloroquine was added to the cells 60 min prior to PPME photosensitization (carried out as in Fig. 2), and nuclear extracts were prepared after 30 min or 24 h. NF-κB induction was measured by EMSA and expressed in fold induction compared with the control (non-photosensitized cells). The intensities of the specific band were measured by phosphorimaging. B, C2-ceramide induces NF-κB in HCT-116 cells. Cells were treated with 30 μM C2-ceramide, and nuclear extracts were analyzed by EMSA after various times (from 0 to 24 h). The specificity of the complex was determined by competition with a 50-fold excess of unlabeled wild-type or mutated probe (data not shown).

be involved in the activation mechanism. However, the role of 1O2 as mediator in NF-κB activation by PPME photosensitization could be considered unlikely because (i) none of the tested antioxidants (hydrophilic and hydrophobic) capable of quenching 1O2 inhibit NF-κB activation and (ii) no lipoperoxide can be detected in photosensitized HCT-116 cells. The increased NF-κB activation by PPME photosensitization after D2O substitution could then be explained by an increased lifetime of the PPME excited state which has already been observed for other photosensitizers (51). This would involve a radical mechanism implicating either an electron or a charge transfer between a PPME excited state and membrane proteins such as the IL-1 type 1 receptor or the acidic SMase. Although a so-called type I reaction would be implicated to explain NF-κB action by PPME photosensitization, we cannot totally rule out that part of the photochemical mechanism could be due to 1O2. Indeed, the lack of inhibition by a lipophilic antioxidant such as vitamin E could also be explained by a subcellular concentration too low to efficiently compete with the reaction between the PPME excited state and membrane receptor. Definitive proof of 1O2 involvement in NF-κB activation could only come from directly measuring its emission at 1268 nm. However, 1O2 detection by infrared emission on cells is currently not feasible.

One of the main contributions of this work is to unambiguously show that PPME photosensitization can specifically mobilize the IL-1 transduction pathway leading to NF-κB activation. This is demonstrated by (i) significant internalization of the IL-1 receptor after photosensitization with PPME; (ii) the inhibition of NF-κB activation by expression of TRAF6 dominant negative, a protein linked to the IL-1 receptor; (iii) the absence of down-regulation by expression of TRAF2 dominant negative mutant protein; and (iv) the increased NF-κB activity when IKK-α, IKK-β, and IKK-α plus -β are overexpressed in photosensitized cells. The type of IL-1 receptor whose internalization is increased by PPME photosensitization is likely to be the type I receptor because the IL-1 type II receptor is not capable of transducing signals and mainly acts as a decoy receptor (60). Interestingly, such an internalization cannot be recorded with the TNF receptor. One explanation could be that PPME cannot promote TNF receptor trimerization, which is required to both transduce signals and promote internalization (61). Whereas the molecular mechanism by which PPME promotes IL-1 receptor internalization is unknown, it could be interesting to determine whether this effect is restricted to receptors that do not require homo- or heterodimerization for their functioning. A modification of cell-surface receptor by PPME photosensitization has recently been published (62); curiously, these authors reported that a mixture between phorbol-12-acetate and PPME can reduce binding of cytokines to their receptors (TNF-α, IL-8, complement factor 5a, and epidermal growth factor). As these data were obtained (i) by measuring cytokine binding to their receptor on neutrophils and (ii) with a mixture of photosensitizers, they reinforce the idea that these compounds can interact with cell-surface receptors and either inactivate them or trigger transduction pathways. The localization of photosensitizers into cellular membranes is therefore expected to be an important determinant controlling the susceptibility of surface proteins. For example, di-hematoporphyrin ether was reported to affect the binding of antibodies to high affinity Fc receptors but not to other surface molecules (63), and methyl pheophorbide-a inhibited the binding of several cytokines to their receptor but not the binding of TNF-α (64). Collectively, these data support the hypothesis that a particular membrane environment, as well as the nature of the receptors, may determine whether the receptor function is activated or inhibited and, therefore, the extent of cell response to PDT.

This new concept is important because it will greatly influence our understanding of the cellular response to PDT not only in terms of tumor cell survival but also of the modification of tumor environment by the release of mediators. As PDT not only reduces tumor burden but also induces inflammation, it is proposed that recruitment of activated macrophages to the inflamed tumor is an important factor in complete tumor eradication (65). In this respect, study of the mechanism of NF-κB activation by PDT is important because many genes encoding cytokines and chemokines are controlled by this factor. Work is now in progress in our laboratory to determine the nature of the genes that are up- and down-regulated by PPME photosensitization. An answer to these questions will lead to a better understanding of the role of host cell response in the antitumor effect of PDT and how the immune response can potentiate antitumor immunity. Recently, it has been demonstrated in a BALB/c mouse model that PDT delivered to normal and tumor tissue in vivo causes marked changes in the expression of IL-6 and IL-10 but not of TNF-α, suggesting that the general inflammatory response to PDT may be mediated by IL-6 (66). Because cytokine genes are controlled by NF-κB and by other transcription factors such as AP-1, c-EBP, CREB, etc., the
differential regulation of cytokine genes by PDT could be due to the better inducibility of several transcription factors as opposed to others which could be weakly or not at all regulated by PDT. For example, Photofrin has been shown to lead to a strong and prolonged activation of c-Jun and c-Fos (67), demonstrating that genes having both NF-κB-and AP-1-responsive elements in their promoter are prone to be up-regulated by PDT.

This paper has shown that NF-κB can be considered as a main transcription factor activated by PDT. Mechanism of PMME-mediated NF-κB activation is rather unique because it resembles the response elicited by IL-1 but differs from the response initiated by ROS generation. Membrane localization of PMME is responsible for this peculiar response; the effect on IL-1 receptor and the rapid NF-κB activation is due to PMME in the cytoplasmic membrane, but the slow and sustained NF-κB activation is likely explained by lysosomal PMME localization giving rise to the production of ceramide by activation of the acidic SMase. As many hydrophobic photosensitizers can target giving rise to the production of ceramide by activation of the cytoplasmic membrane, but the slow and sustained NF-κB activation of mouse lymphoma cells (68), demonstrating that genes having both NF-κB and prolonged activation of c-Jun and c-Fos (67), demonstrating that genes having both NF-κB and prolonged activation of c-Jun and c-Fos (67), demonstrating that genes having both NF-κB and prolonged activation of c-Jun and c-Fos (67).