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Immunological Detection of *N*-formylkynurenine in Porphyrin-Mediated Photooxided Lens α-crystallin

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Abstract

Crystallin proteins are responsible for maintaining lens transparency and allowing the lens to focus light undistorted onto the retina. The α-crystallins are the major lens crystallins, and function as both structural proteins and chaperones to protect all lens proteins from damage leading to lens deterioration. Because lens crystallin proteins do not turn over, the damage they accumulate can lead to cataracts, the world's leading cause of blindness. Photosensitizing porphyrins can accumulate in the eye through either endogenous metabolism or through therapeutic or diagnostic procedures. Porphyrin buildup exacerbates lens aging through increased levels of singlet oxygen, resulting in protein polymerization and amino acid residue alteration. Tryptophans oxidize to kynurenine and *N*-formylkynurenine (NFK) causing irreversible changes in the refractive index of the normally transparent lens, leading to development of cataracts. Additionally, NFK is itself a photosensitizer, and its presence exacerbates lens deterioration. This work uses anti-NFK antiserum to study porphyrin-facilitated photooxidation of α-crystallin tryptophan residues. *In vitro* experiments show that four biologically interesting porphyrins mediate α-crystallin polymerization and accumulation of both protein radicals and NFK. Confocal microscopy of cultured human lens epithelial cells indicates that while all four porphyrins photosensitize cellular proteins, not all oxidize the tryptophans of cellular α-crystallin to NFK.

INTRODUCTION

The predominant proteins of the vertebrate eye lens are the crystallins, with α-crystallin comprising up to 40-50% of all lens protein (1,2), and the two similar subunits, αA and αB, occurring in a ratio of approximately 3:1. While both subunits are found in other organs, αB-crystallin is far more ubiquitous, acting as a heat shock protein/chaperone in the lens and in other tissues (1,2). Alpha-crystallin, thus, plays a dual role in the lens: 1) It assembles into an orderly arrangement of protein fibers that results in a transparent lens and 2) It acts as a chaperone to maintain the integrity of all lens proteins, including itself and the other crystallins (1,2). Interest in the role of α-crystallin as a chaperone, combined with studies of protein aggregation in disease development, have lead to the observation that increased levels of αB-crystallin are associated with neurological disorders such as Alexander's disease, Creutzfeldt-Jacob disease, Alzheimers, Parkinsons (2).

The human lens continues to grow throughout our lifetime, although it grows more slowly with age. Progenitor lens epithelial cells differentiate into lens fiber cells which, at maturity,

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lack all intracellular membrane-bound organelles (3). Although new lens proteins continue to be produced, α-crystallin lens proteins do not turn over, and the lens retains all the α-crystallin it has synthesized. Damage to these essential proteins, therefore, is cumulative and can result in the buildup of altered amino acid residues leading to deleterious alterations in the refractive index and ultimately opacification (cataractogenesis) of the normally transparent lens (4). Despite advances in lens replacement technology, the cataracts thus produced remain the leading cause of blindness in the US and the world (5).

Porphyrins can accumulate in the eye and in other organs due to metabolic errors or when injected for diagnosis of cancer or as part of photodynamic cancer therapy (6-8). Porphyria cutanea tarda, for example, is a disorder resulting from low levels of one of the heme biosynthetic enzymes, leading to the buildup in the blood of the heme porphyrin intermediate uroporphyrin III (9). The subsequent dermal accumulation of this porphyrin leads to increased photosensitivity (10). Along with the skin, the eyes are the organs most exposed to light, and therefore the skin and the eyes are the organs most likely to suffer photooxidative damage. Although the cornea cuts off all radiation below 295 nm, this still allows wavelengths capable of photosensitizing porphyrins to reach the lens, which absorbs radiant energy between 295 nm and 400 nm. *In vitro* studies with different porphyrins have shown them to be efficient generators of singlet oxygen ($^1O_2$) which can cause polymerization of α-crystallin and can react with and modify amino acid residues (11). Additionally, some porphyrins can bind to lens proteins, thereby enhancing the potential for harmful interactions (11-13).

Photosensitized protein damage is understood to occur through two major and distinct routes. Type I reactions involve direct ionization of the protein through absorption of UV radiation by the protein itself or by a bound or adjacent chromophore, resulting in the generation of radicals. Type II, indirect, reactions occur via an energy transfer from the excited chromophore to molecular oxygen, resulting in the production of $^1O_2$, which then reacts with the protein. The differentiation, however, between, radical (Type I) and non-radical, $^1O_2$-mediated (Type II) reactions resulting from photosensitization is complicated by the long-lived hydroperoxides that can be produced in $^1O_2$-exposed proteins and cells (14,15). Metal ions and UV radiation can catalyze the decomposition of these hydroperoxides to radicals (16,17) thereby increasing the potential for radical-mediated, but non-Type I, interactions (14,15,18).

While tyrosine, cysteine and methionine also react with $^1O_2$ at biologically relevant rates, histidine and tryptophan have the highest $^1O_2$ rate constants for reaction with protein side-chains at physiological pH (17). Uniquely amongst the amino acids, tryptophan can act as both a physical and chemical quencher of $^1O_2$, with the relative contributions of the two routes being affected by various factors (16). Reaction products of tryptophan and tryptophan residues with $^1O_2$ include hydroperoxides, hydrotryptophans, kynurenine and *N*-formylkynurenine (NFK). Identification of tryptophan, and other, oxidation end-products frequently involves time-consuming and technologically intense methods such as mass spectrometry (19-21) and HPLC (22,23) for identification of stable end-products. Unfortunately, the sample handling required for these kinds of analyses has the potential to generate artifacts (24), producing misleading data.

We recently published a more generally approachable, less technologically complex immunological method for detection of NFK residues in proteins (25). The use of our rabbit polyclonal anti-NFK antiserum has allowed us to detect NFK in photooxidized single proteins and protein mixtures, in human SOD1 oxidized by carbonate anion radical and to visualize NFK in cultured human HaCaT kerotinocyte cells (25,26).

In this study we investigate the role of NFK accumulation in the photobiology of cataract development. As photosensitizing agents we chose porphyrins that are of biological interest due to their roles in either a disease state (porphyria cutanea tarda) or their potential as agents for photodynamic therapy (6-9). We used UVA radiation to examine porphyrin photosensitizing effects on the accumulation of NFK in α-crystallin *in vitro* and in human lens epithelial (HLE) cell culture because UVA wavelengths best overlap those wavelengths absorbed by the human lens. *In vitro* photosensitization was performed in both the presence and absence of the $^1O_2$ quencher azide and the radical spin trap/scavenger DMPO. Confocal microscopy was used to assess the extent of NFK colocalization with α-crystallin in photosensitized human epithelial cell culture.

MATERIALS AND METHODS

Materials

Bovine α-crystallin, TPPS (mesotetra (*p*-sulfonatophenyl) porphyrin) and uroporphyrin were purchased from Sigma Chemical Co. (St. Louis, MO). Ce6 (chlorin e6) and THPP (mesotetra (m-hydroxyphenyl) porphine) were purchased from Porphyrin Products (Logan, UT). Stock solutions of 1 mM TPPS, Uro and Ce6 were made in chelex-treated, 100 mM phosphate buffer, pH 7.4, while a 1 mM stock solution of THPP was dissolved in 95% ethanol. The structure of the four porphyrins used in this study are shown in Fig. 1.

UVA irradiation

Solutions (0.5 ml) of 1 mg/ml bovine α-crystallin in chelex-treated, 100 mM phosphate buffer, pH 7.4, were placed in individual wells of a 24-well microtiter plate (Corning Incorporated, Corning, NY) with and without the addition of 1 or 10 μM porphyrin. The samples were then either kept in the dark (0 min, 0 J cm^{-1}) or were irradiated with UVA for 10, 20 or 40 min at irradiances of 2.5, 5.0 and 10 J cm^{-1}, respectively, as measured with a YSI-Kettering Model 65A Radiometer (Yellow Springs Instrument Co., Yellow Springs, OH). The UVA radiation source was composed of four parallel fluorescent UVA lamps (Houvalite F20T12BL-HO; National Biological Co. Twinsburg, OH). The emission spectrum of the UVA radiation was measured with a spectroradiometer (LuzChem Research, Inc., Ottawa, ON, Canada) (Fig. 2). Parallel experiments were performed with inhibitors and spin trap using 10 mM sodium azide, 200 mM DMPO individually or in combination.

Protein electrophoresis and western analysis

Duplicate gels containing 13 μg aliquots of α-crystallin were electrophoresed under reducing conditions. One gel of each pair was stained with Coomassie blue while the other was transferred to nitrocellulose for western analysis. Anti-NFK westerns were performed as previously described (25,26). Anti-DMPO westerns were performed using 10 μg/ml chicken polyclonal anti-DMPO (27).

Spectroscopic measurements

Absorption spectra were recorded on a Cary 100-Bio UV visible spectrophotometer. Fluorescence measurements were done on a FluoroLog 3 spectrofluorometer (Horiba Jobin Yvon, Edison, NY). Four hundred nm was chosen as the excitation wavelength because all samples absorb strongly at that wavelength.

Cell culture

An extended lifespan human lens epithelial cell line (HLE B-3) was used in these studies (28). Cells were grown in Eagle's MEM (Sigma) containing 2 mm l-glutamine, 50 μg mL^{-1}

gentamicin and 20% FBS in an atmosphere of 5% CO_2/95% air at 37°C. Cells were fed three times a week and after attaining confluence were passaged using trypsin (0.125%)-EDTA (0.5 mM). For experiments with HLE cells, the cells were incubated for 18 h at 37°C in the dark with porphyrin photosensitizer, and, after washing with PBS were irradiated with UVA for 15 min (3.75 J cm^{-1}) after which sodium azide was added to a final concentration of 10 mM.

Confocal microscroscopy

HLE B-3 cells (28) were seeded onto 35 mm plates containing 1.5 mm thick coverslips (MatTek, Ashland MA) and grown to near confluence. Control and treated cells were fixed and then stained simultaneously with rabbit polyclonal anti-NFK (1:250 dilution) and both mouse monoclonal anti-αA-crystallin and mouse monoclonal anti-αB-crystallin (1:500 dilutions) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). After washing the cells were then stained simultaneously with 1:1000 dilutions of Alexa Fluor 488 anti-mouse and Alexa Fluor 568 anti-rabbit antibodies. For visualization of nuclei, cells were stained for 10 min in a solution of 1 μg/ml DAPI (Thermo Scientific, Rockford, IL).

RESULTS

Photosensitization of bovine α-crystallin

Bovine α-crystallin was mixed with 1 or 10 μM porphyrin photosensizer (Fig. 1) and exposed to increasing durations of UVA irradiation (0, 10 min, 20 min, 40 min). Control (porphyrin minus) aliquots of protein were also irradiated for 40 min with UVA. Samples were then subjected to SDS-PAGE electrophoresis for parallel examination of subunit cross-linking by Coomassie blue staining and tryptophan residue oxidation by anti-NFK western blotting. Coomassie staining of the samples showed that photosensitization with all four porphyrins resulted in polymerization of α-crystallin (Fig. 3, top panel). TPPS and Uro were most efficient in promoting photo-cross linking of lens protein, forming trimers and higher molecular weight polymers at a concentration of 1 μM after only 10 or 20 minutes of UVA exposure. Both THPP and Ce6 also promoted polymerization to high molecular weight polymers, but required more extensive photosensitization, achieved through either a higher concentration of porphyrin or longer UVA exposure.

The anti-NFK western blots roughly parallelled the Coomassie gels (Fig. 3, bottom panel). The efficacy of α-crystallin photosensitization as measured by tryptophan conversion to NFK (TPPS>Uro>Ce6>THPP) corroborates the results from protein staining (Fig. 3). Where there is little or no cross-linking, as in the UVA only and the dark/photosensitizer controls, there is little or no detectable NFK. NFK accumulation was detected in dimers, trimers and higher molecular weight α-crystallin polymers in all or nearly all the bands seen in the Coomassie-stained gels. And, with the exception of the Uro-photooxidized samples, NFK was also present at above background levels in at least some of the α-crystallin monomers.

Parallel irradiations were also performed in the presence of the $^1O_2$ quencher sodium azide, the radical scavenger/spin trap DMPO, or both (Fig. 4). This experiment used only the maximum photosensitization conditions used in Fig.1, i.e. 10 μM porphyrin and 40 min UVA irradiation. Coomassie staining of SDS-PAGE gels (top panel) showed that, although neither azide nor DMPO alone or in combination completely inhibited photo-induced cross-linking, azide was somewhat more effective than DMPO.

Anti-NFK blots western blots (Fig. 4 middle panel) showed that the effect of azide on NFK accumulation generally echoed that of the effect of azide on cross-linking. Azide reduced NFK accumulation in the Ce6 and THPP samples, but produced little, if any, decrease in the TPPS samples. The Uro reactions were most drastically effected, with NFK accumulation

becoming undetectable. Anti-DMPO westerns (Fig. 4, bottom panel) showed that addition of DMPO allowed the trapping of α-crystallin radicals in all photosensitized samples. These western blots also showed that supplementation of azide to the DMPO-containing, Uro-photosensitized samples substantially reduced radical adduct production. Azide supplementation of the comparable DMPO-containing, Ce6- and THPP-photosensitized samples resulted in a less marked, but still detectable, decrease in DMPO adduct accumulation.

Absorption and fluorescence spectra of porphyrins with and without α-crystallin

Binding of porphyrins to α-crystallin would increase their potential for producing $^1O_2$-mediated protein damage. To assess binding between the porphyrins in this study and α-crystallin we determined porphyrin absorption and fluorescence spectra in the absence and presence of the protein. The absorption spectra of the 1 μM porphyrin samples, both with and without 1 mg/ml α-crystallin, are shown in Fig. 5. The presence of the lens protein has no effect on the absorption peak of either Ce6 or Uro, while the absorption peak of TPPS is slightly red shifted in the presence of α-crystallin, consistent with previously published work (11). The absorption spectrum of THPP is most altered by the presence of α-crystallin, with a large blue shift and both a narrowing and heightening of the peak. Fluorescence emission spectra of the same samples excited at a wavelength of 400 nm are shown in Fig. 6. While the fluorescence spectrum of Uro is unaffected by the presence of α-crystallin protein the spectra of the other porphyrins are altered in both amplitude and emission maxima. The emission spectrum of TPPS is both reduced and the peak red-shifted. Both Ce6 and THPP show enhanced fluorescence in the presence of α-crystallin, with the emission peak of Ce6 being slightly red-shifted while the emission spectrum of THPP is very slightly blue-shifted.

Detection of NFK in HLE cells and colocalization with α-crystallin

HLE cells were incubated for 18 h in the dark with porphyrin photosensitizers, and after washing with PBS were exposed to UVA or were kept in the dark. After fixation the cells were stained with anti-α-crystallin (green), DAPI (blue), and anti-NFK (red) (Fig. 7A-dark and Fig. 7B-UVA). As expected, all of the cells regardless of porphyrin treatment or UVA exposure contain obvious cytoplasmic accumulations of α-crystallin. Also as expected, the "dark" cells contain little detectable NFK. The porphyrin-containing, UVA exposed cells, however, clearly contain NFK. The Uro-treated cells accumulate the lowest amount of NFK, followed by cells treated with TPPS and Ce6 and THPP. While the UVA irradiated TPPS and Uro treated cells accumulate NFK, only the Ce6 and THPP treated cells show clear and extensive colocalization of NFK with α-crystallin.

DISCUSSION

This work used a combination of *in vitro* and cell culture experiments to study the effect of four biologically interesting porphyrins (Fig. 1) and UVA exposure on lens α-crystallin. Coomassie staining showed that neither UVA radiation alone nor addition of porphyrin alone results in α-crystallin polymerization (Fig. 3). Increasing photosensitization, however, by either increased UVA exposure in the presence of a porphyrin or increased porphyrin concentration in the presence of UVA radiation, does result in more extensive protein polymerization. The NFK westerns show that NFK accumulation parallels protein polymerization, which also increases with increasing photosensitization. Of the porphyrins used, TPPS was the most potent photosensitizer *in vitro*, causing both more extensive polymerization and NFK accumulation at the lowest concentration of porphyrin and shortest duration of UVA exposure.

To differentiate between the contributions of Type I (radical) and Type II ($^1O_2$) reactions to the protein modifications observed in Fig. 3 we used the $^1O_2$ quencher sodium azide and the radical scavenger/spin trap DMPO (Fig. 4). Azide reduced cross-linking of α-crystallin more effectively than DMPO, indicating that Type II reactions were primarily responsible for increased polymerization of the protein. Decrease in NFK accumulation roughly paralleled the differences in cross-linking, again suggesting that $^1O_2$ was responsible for tryptophan residue conversion to NFK. The anti-DMPO western blots, however, showed that Type I reactions also play a role in porphyrin-mediated photosensitization of α-crystallin. Radicals are trapped by DMPO in all photosensitized samples, and, in the samples irradiated in the presence of Uro, radical trapping/scavenging reduces both cross-linking and NFK accumulation. In addition to Type I radical-mediated events there is also evidence for the production of radicals from $^1O_2$ generated protein peroxides which can, in the presence of metals or UV radiation, decompose to radicals (16,17). This UV radiation catalyzed decomposition of protein peroxides to radicals could explain the decrease in DMPO radical adduct production seen in photosensitized samples containing both DMPO and azide (Fig. 4, bottom panel).

Absorbance (Fig. 5) and fluorescence (Fig. 6) spectra were obtained to assess binding between protein and porphyrin. The absorbance and fluorescence maxima of both TPPS and THPP are shifted in the presence of α-crystallin, indicated binding between porphyrin and protein. Ce6 appears to participate in a less robust interaction with α-crystallin, as the spectra are altered in amplitude but show a less pronounced shift in wavelength. Uro, however, appears to have no interaction with α-crystallin as neither spectrum is altered in the presence of the protein (Figs. 5 and 6) (11). Porphyrin interaction with α-crystallin influences the extent of $^1O_2$-mediated polymerization seen in our *in vitro* experiments (Figs. 3 and 4). The porphyrins that interact the most strongly with the protein (TPPS and THPP) are also more resistant to azide quenching.

Recently, Medinas et al (29) described the characterization of a tryptophan dimer resulting from the carbonate-dependent peroxidase activity of human SOD1 (30,31). Interestingly, NFK formation and this novel mode of cross-linking and appear mutually exclusive. When they used reaction conditions focused on optimizing hSOD1 dimer yield Medinas et al (29) detected no NFK in the dimer. Additionally, our original publication describing the anti-NFK antibody (25) showed that while increasing H_2O_2 concentration up to 10 mM increased NFK accumulation in SOD1, dimer formation was maximal at 0.5 to 1.0 mM H_2O_2. It is unlikely, therefore, that the protein polymers seen in Fig. 3 are due to tryptophan dimerization. There is, however, evidence that dityrosine formation occurs in crystallin proteins via a radical-mediated process (32). And, in addition to the 8 tyrosine residues found in the two subunits of α-crystallin, there are also a total of 16 histidine residues which can form His-His and His-Lys crosslinks (16).

The confocal microscopy analysis (Fig. 7) shows that in HLE cells the extent of porphyrin photosensitization is further influenced by their differential subcellular localization. In this experiment, while all four porphyins promote accumulation of NFK, substantial NFK accumulation that colocalizes with α-crystallin is only present in Ce6- and THHP-photosensitized cells. Thus, while TPPS promotes abundant oxidation of tryptophan to NFK *in vitro*, in HLE cells, where the acidic TPPS accumulates in lysosomes (33,34), there is little interaction between the porphyrin and the protein and therefore less photosensitized damage. THPP and Ce6, on the other hand, localize in HLE cells in the same compartment as α-crystallin, enabling physical interactions between protein and porphyrin, allowing opportunity for extensive NFK accumulation in α-crystallin protein molecules. The capacity of each porphyrin to photosensitize α-crystallin is a result of a combination of factors,

including absorption of radiant energy, $^1O_2$ quantum yield, and ability to bind to α-crystallin and/or to co-localize with α-crystallin in HLE cells.

Previous studies have examined photosensitization of α-crystallin by porphyrins and other biologically relevant substances (11,20-22,35). Porphyrins (TPPS, Uro, hematoporphyin) and other photosensitizers such as hypericin, an ingredient of the herb St. John's wort used to treat depression, and xanthurenic acid, a metabolite of tryptophan, all facilitated polymerization of α-crystallin under UVA irradiation and also promoted the oxidation of specific amino acid residues such as histidine, methionine, tyrosine and tryptophan. In this work we demonstrated that the porphyrin-mediated photooxidation of α-crystallin that leads to protein polymerization also leads to accumulation of NFK in monomers, dimers and higher molecular weight species. Additionally, we were able to detect the oxidation of cellular tryptophan residues to NFK in human lens epithelial cells, and to visualize the colocalization of NFK with α-crystallin in photosensitized cells.

The absorption spectrum of the human lens changes with age. The adult human lens absorbs most light and radiation between wavelengths of 295 to 400 nm due to the presence of 3-OH kynurenine and its glucoside, (36,37) while the aged human lens absorbs longer wavelengths of light into the blue visible region due to a change in lenticular chromophores (20). Photosensitizers which absorb wavelengths of light above 400-450 can damage both the lens and retina. Because NFK is itself a $^1O_2$-generating photosensitizer, accumulation of NFK in lens proteins serves to exacerbate the potential for light-mediated damage. Furthermore, the long-lived triplet state of a photosensitizer can react with reducing substrates to produce radicals in type I reactions (18), and these radicals are also capable of altering amino acid side chains as well as the protein backbone.

Although the other eye lens crystallins (beta- and gamma-crystallins) represent a smaller percentage of the total protein of the lens, they are also susceptible to oxidation of tryptophan residues to NFK (38). Because α-crystallin is a chaperone protein in the lens and other tissues, these observations may have implications that extend beyond eye functioning and health to areas related to protein folding and stress physiology. Indeed, there has been growing interest in the effect of post-translational modifications on α-crystallin efficiency as a chaperone (39-42). Abraham et al (39) observe an inverse correlation between glycation of lysine residues of and chaperone efficiency. The availability and accessibility of NFK immunological detection will facilitate the unraveling of the potential effect of tryptophan residue oxidation to NFK on chaperone activity.

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In this study we used immunological techniques to detect *N*-formylkynurenine (NFK), a tryptophan oxidation product, in α-crystallin using an anti-NFK antibody. Here we show UVA irradiated human lens epithelial cells containing the porphyrin THPP (mesotetra (m-hydroxyphenyl) porphine). Fluorescent confocal analysis was used to visualize (clockwise, starting at bottom left) nuclei (blue), NFK (green), and α-crystallin (red), the major lens protein. The final panel (bottom, right) shows the overlay image of the preceding three, and illustrates the potential for irreversible oxidative photodamage that porphyrin accumulation can inflict on the critical eye lens protein α-crystallin. Unirradiated porphyrin-containing cells do not accumulate NFK.

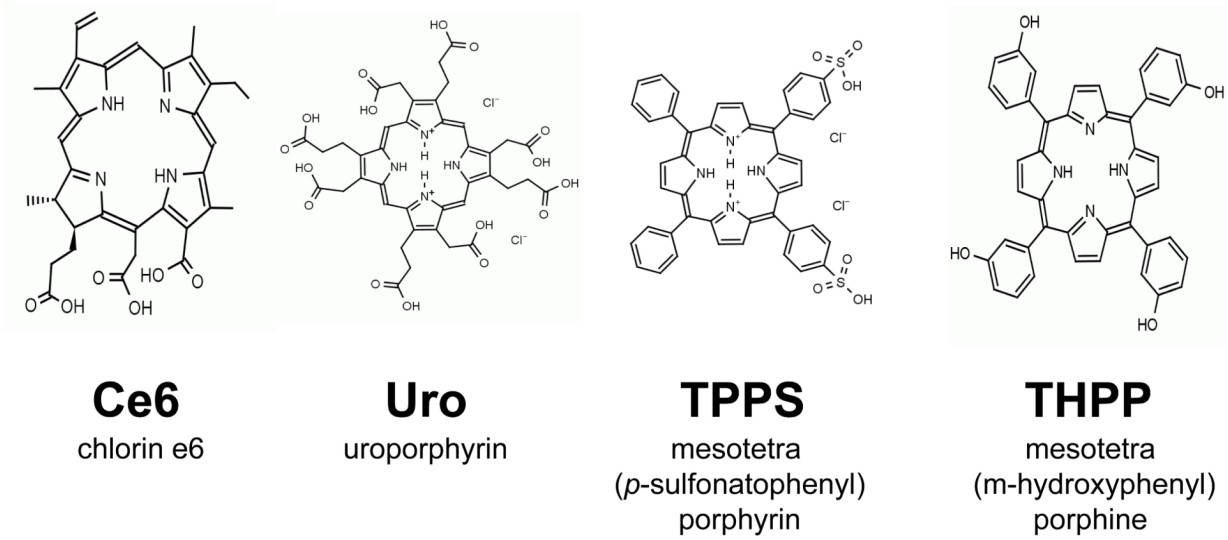


Figure 1.
Chemical structure of porphyrins used in this study.

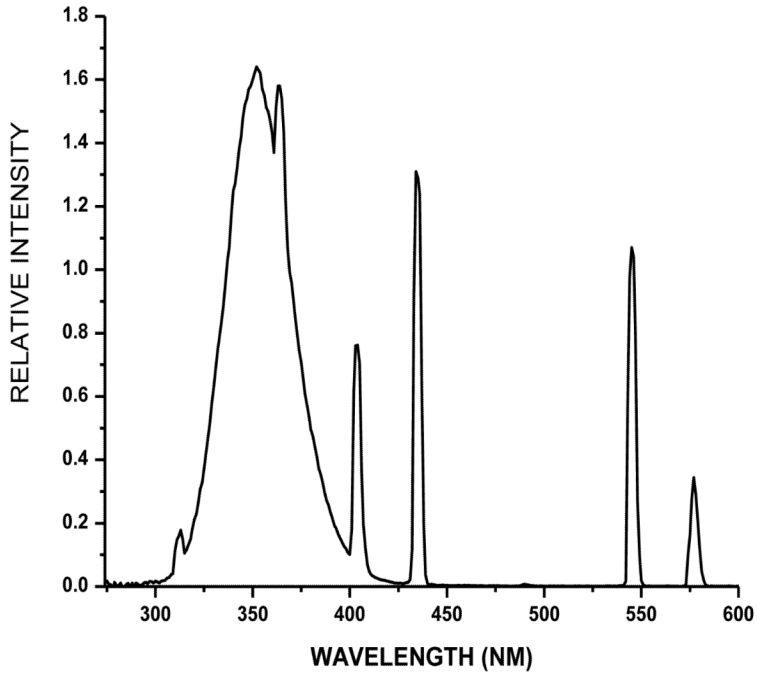


Figure 2.
Emission spectrum of UVA radiation source with four parallel fluorescent UVA lamps.

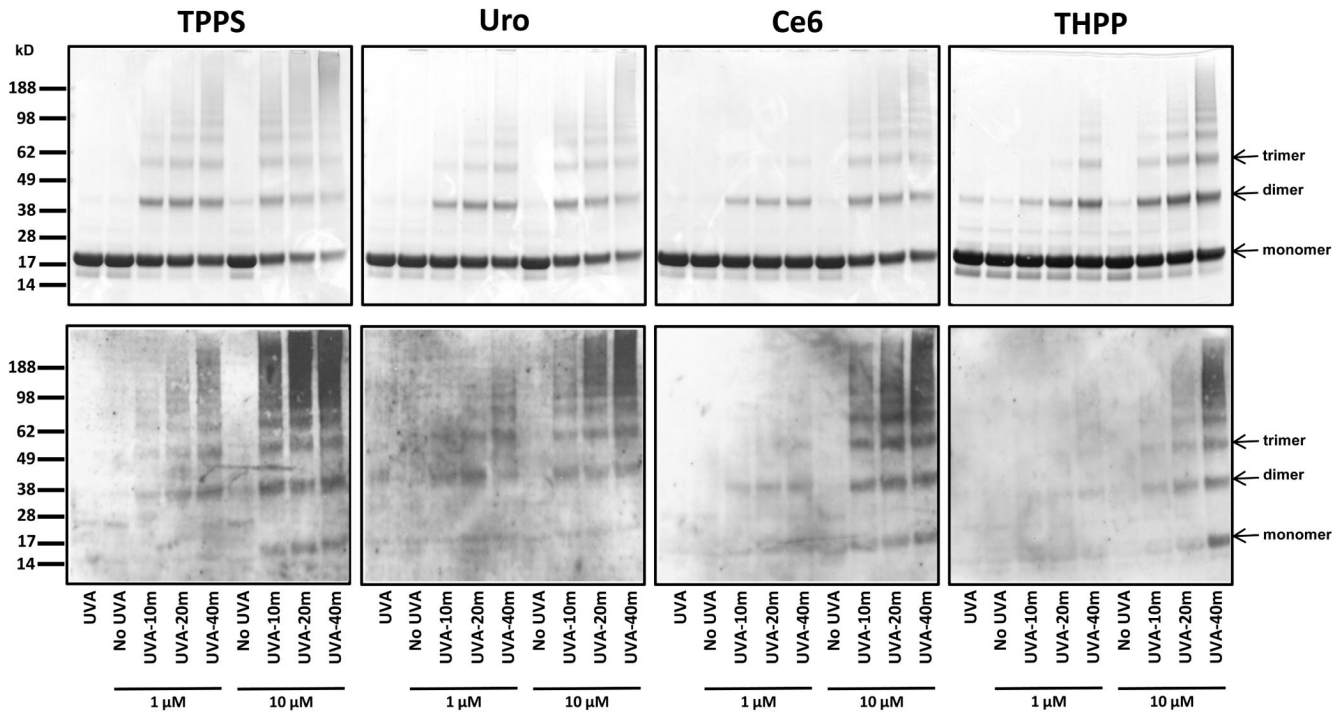


Figure 3.

Photosensitization of α-crystallin by porphyrins promotes cross-linking and accumulation of NFK. Aliquots of α-crystallin were incubated without addition of porphyrin or with 1 μM and 10 μM of the indicated porphyrins and were either kept in the dark (no UVA) or were exposed to 10, 20 or 40 min of UVA irradiation as described in the materials and methods. For each porphyrin duplicate gels containing 13 μg of protein per lane were subjected to SDS-PAGE electrophoresis under reducing conditions and one gel was stained with Coomassie blue (top panels) and the other was transferred to nitrocellulose for western analysis with anti-NFK antiserum (bottom panels).

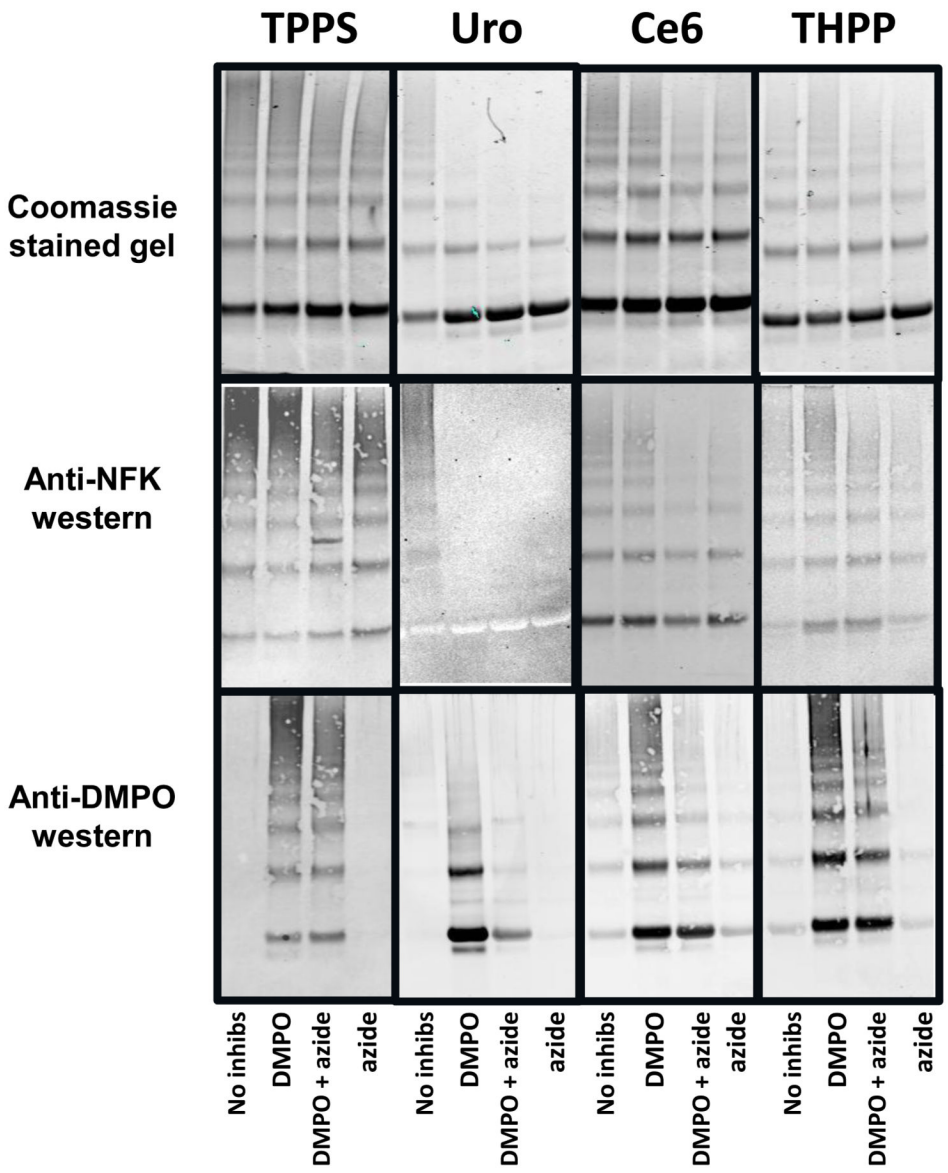


Figure 4.

Photosensitization of α-crystallin by porphyrins in the presence of the singlet oxygen quencher azide and the radical spin trap/scavenger DMPO. Aliquots of α-crystallin containing 10 μM of the indicated porphyrin, and containing either no further additions or 10 mM NaN_3 or 200 mM DMPO or both, as indicated, were exposed to 40 min of UVA irradiation as described in the materials and methods. For each porphyrin three duplicate gels containing 13 μg of protein per lane were subjected to SDS-PAGE electrophoresis under reducing conditions. One gel was stained with Coomassie blue (top panels), and two were transferred to nitrocellulose for western blot analysis with anti-NFK (middle panels) and anti-DMPO (bottom panels) antisera

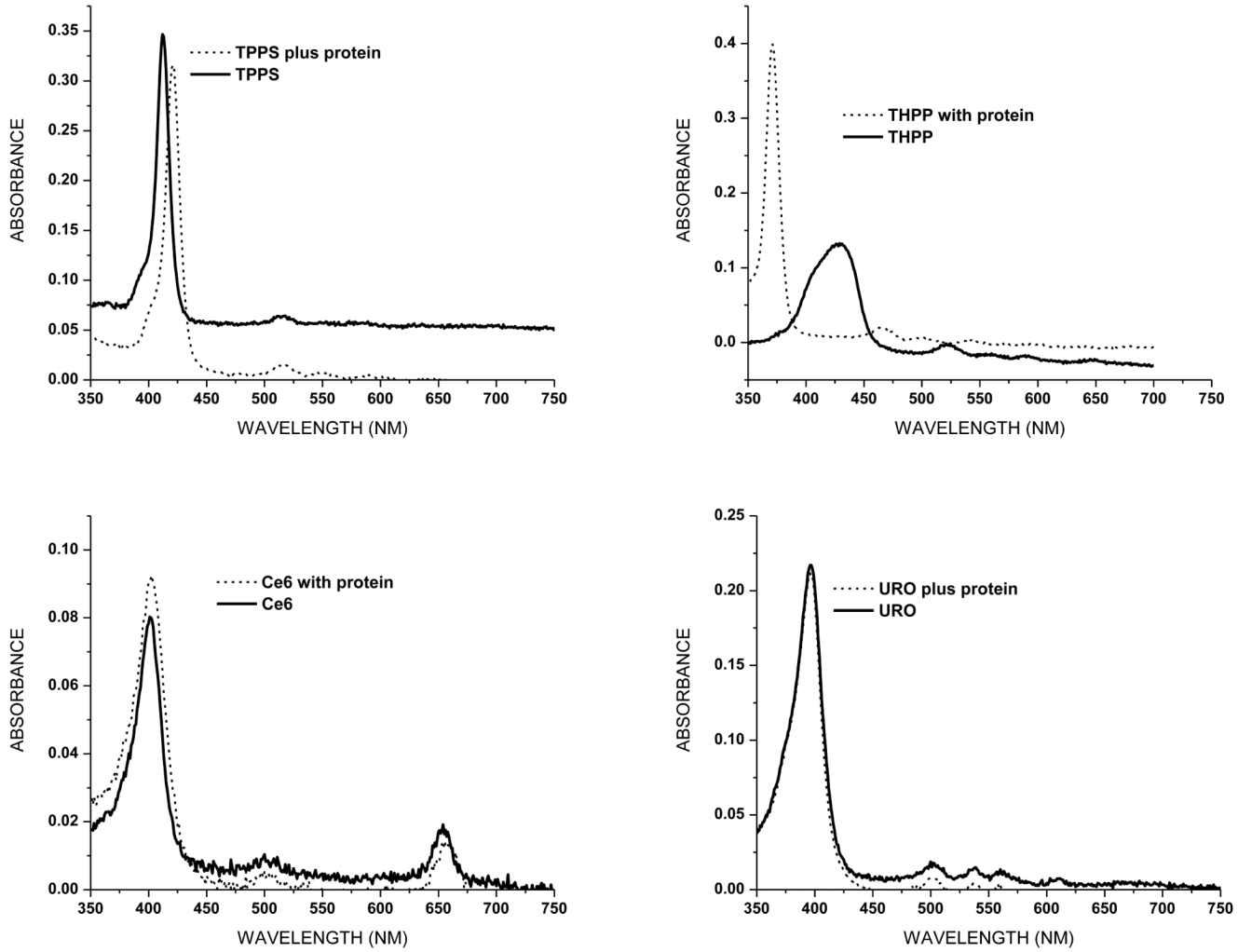


Figure 5.

Absorption spectra of porphyrins with and without 1 mg/ml α-crystallin. A solution of 1 mg/ml was used to zero the spectrophotometer.

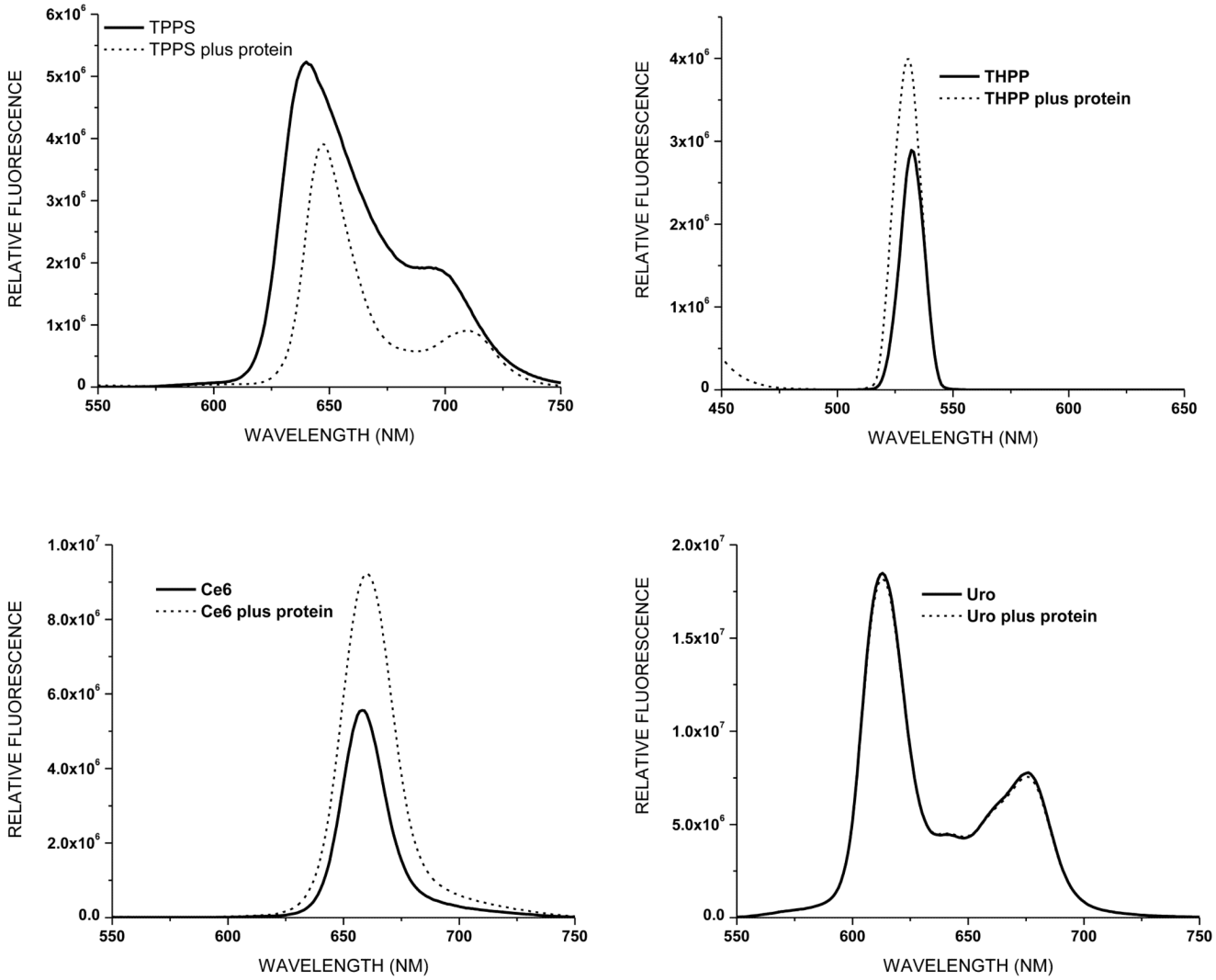
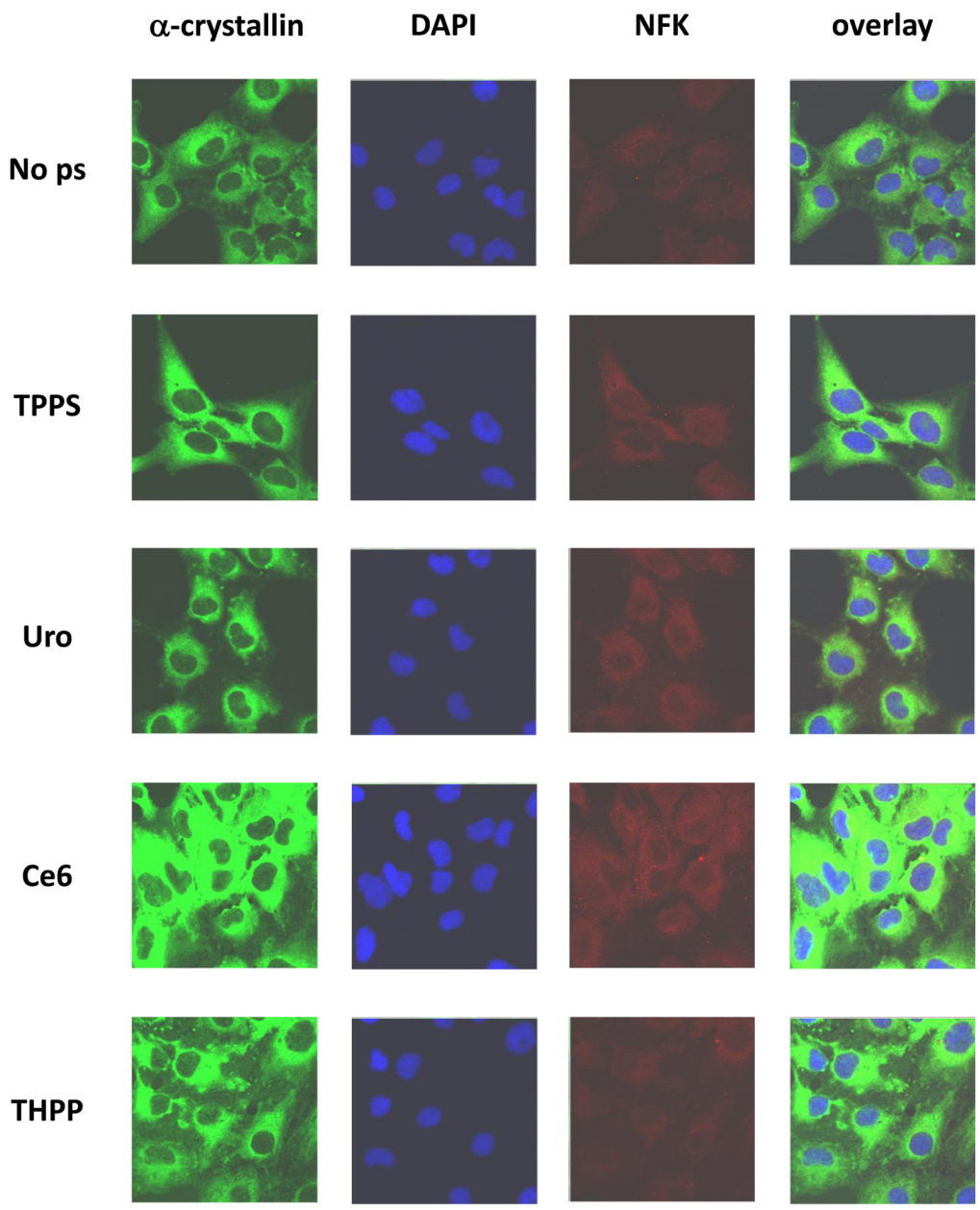


Figure 6.
Fluorescence spectra of porphyrins with and without 1 mg/ml α-crystallin. Samples were excited at a wavelength of 400 nm.

A.



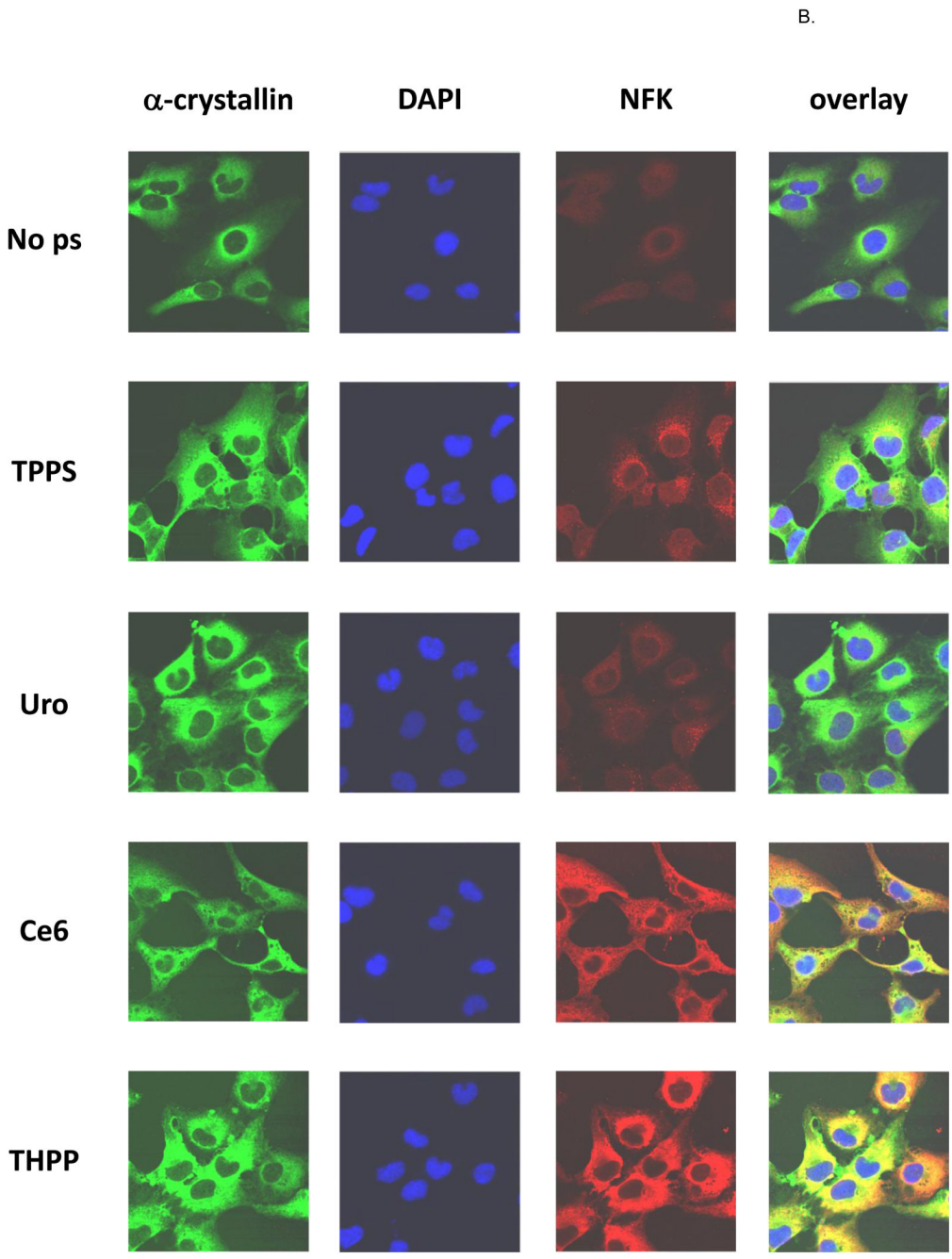


Figure 7.

Confocal fluorescent microscopy of (A) dark-incubated and (B) UVA-irradiated HLE cells. Nearly confluent HLE-B3 cells were treated overnight with or without 10 μM porphyrins as indicated and after washing were kept in the dark. Fixed cells were simultaneously stained with rabbit anti-NFK and mouse anti-αA and anti-αB-crystallin followed by staining with Alexa Fluor anti-rabbit 568 and anti-mouse 488 and then with DAPI. α-crystallin (green), DAPI (blue), NFK (red), and overlay of all three.