in vivo *23*: 281-286 (2009)

Formation of Heme-iron Complexes with Nitric Oxide (NO) and Peroxynitrite ($ONOO^-$) after Ultraviolet Radiation as a Protective Mechanism in Rat Skin

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Abstract. *Ultraviolet C (UVC)-irradiated microvessels isolated from rat skin release free nitrogen radicals, i.e. nitric oxide (NO), peroxynitrite ($ONOO^-$) and nitrosocompounds formed from L-arginine. During UVC radiation of microvessels, heme (Fe^{3+}) is released from hemoglobin and reacts with NO to form nitrosyl-heme (Fe^{2+}-NO). The hydroxyl radical ($OH^•$) produced is attached to heme-iron (Fe^{3+}) to form hematin. $ONOO^-$ then binds to Fe(OH) and the complex $Fe[(OH)ONOO^-]^{2-}$ is formed. Thus, in cases of increased oxidative stress, the free heme can act as an endogenous scavenger of $OH^•$ and $ONOO^-$. Furthermore, Fe-NO and $Fe[(OH)ONOO^-]^{2-}$ can act as NO donor and as antioxidant in redox cyclic iron-centered heme reactions, respectively. The scavenging-antioxidant properties of heme complexes, which allow it to protect the cells from the cytotoxic effects of the oxygen and/or nitrogen free radicals, were verified by estimating the changes in membrane fluidity of microvessels after UVC radiation. The present study indicates that UVC radiation of the skin acts as a potent stimulator for the formation of Fe-NO and $Fe[(OH)ONOO^-]^{2-}$ in microvasculature with cytoprotective effects.*

Photoaging is a term presently used to describe the changes in appearance and/or function of human skin as a result of repeated exposure to sunlight especially with regards to wrinkles and other changes in the appearance of the skin. Solar radiation reaching the earth's surface comprises ultraviolet (UV) (λ<400 nm), visible (400 nm < λ <700 nm) and infrared (IR) (λ>700 nm). UV radiation is generally divided into UVA (320-400 nm), UVB (290-320 nm), and UVC (<290 nm); UVC radiation is blocked from reaching the earth's surface by stratospheric ozone (1). Photoaging is characterized clinically by coarseness, wrinkles, mottled pigmentation, shallowness, laxity, eventually premalignant and ultimately malignant neoplasms. It commonly occurs in skin that is habitually exposed to sunlight, such as the face, ears and bald areas of the scalp, neck, forearms and hands (2). During the past 50 years, considerable information has been gathered regarding the relationship of solar radiation to non-melanoma skin cancer in humans. Ultraviolet radiation can also activate *c-fos* gene expression in cells (3).

The skin aging process can be divided into intrinsic aging and photoaging where cutaneous blood vessels undergo significant alterations. Damage of the cutaneous microvasculature has been observed in the skin of individuals exposed to UVC, infrared rays and heat. These stimuli are known to induce skin angiogenesis (4). Moreover, targeted overexpression of vascular endothelial growth factor (VEGF)-A enhances the sensitivity to UVB-induced cutaneous photodamage, indicating that the cutaneous blood vasculature plays a critical role in the mediation of photodamage (5).

Although there is general agreement that NO is involved in inflammation, the literature is confusing, as both proinflammatory and antiinflammatory properties have been ascribed to NO (6, 7). In inflamed tissue NO reacts quickly with O_2^- and as a consequence the toxic peroxynitrite ($ONOO^-$), which promotes lipid and sulfydryl oxidation, is formed (8). $ONOO^-$ on the other hand activates soluble guanylate cyclase producing a vasorelaxant effect. This indicates that the mechanism of O_2^- inactivation of NO is by converting it to a shorter-lived and less potent vasorelaxant species (9, 10).

In the present study, it was demonstrated that microvessels isolated from rat skin released NO and O_2^- to form $ONOO^-$. UVC radiation augmented NO and $ONOO^-$ by increasing NO^-synthase and xanthine oxidase activities. It is also shown

Abbreviations: NO, Nitric oxide; $ONOO^-$, peroxynitrite; SOD, superoxide dismutase; UVC, ultraviolet C; L-NMMA, *N*-monomethyl-L-arginine; Fe-NO, nitrosyl-heme; TMA-DPH, 1,4-trimethylamino-phenyl-6-1,3,5,hexatriene.

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Key Words: Nitric oxide, peroxynitrite, heme, nitrosyl-heme, ultraviolet C radiation, skin microvessels, erythema, inflammation, tumor.

that in UVC-irradiated microvessels, heme was released from hemoglobin. The heme-iron was reduced by NO to form nitrosyl-heme (Fe-NO). Hematin (Fe-OH) bound to $ONOO^-$ and formed the complex $Fe[(OH)ONOO^-]^{2-}$. Thus, heme can act as a scavenger of $OH^\bullet$ and $ONOO^-$ and therefore as a protective as well as defensive agent against the damaging effects of $OH^\bullet$ and $ONOO^-$. On the other hand, after UVC-induced damage, Fe-NO can act as an NO donor, assisting the remodeling of the skin.

Materials and Methods

Materials. NO (99.99% pure) was obtained from Messer Griesheim (Germany); Hemin hydrochloride, hydroxylamine and 1,4-trimethylamino-phenyl-6-1,3,5,hexatriene (TMA-DPH) were obtained from Sigma Chemical Co (St. Louis, MO, USA); arginine L-NMMA and superoxide dismutase (SOD) were obtained from Calbiochem (Switzerland). Other reagents, solvents and salts were of analytical grade and were obtained from Sigma Chemical Co.

Microvessel preparation from rat skin. Rat skin microvessels were isolated by a method described by Tayarani with a slight modification (11). Rat skin was homogenized (10% w/v) in a pH 7.4 buffer containing 135 mM NaCl, 4 mM KCl, 3.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.5 mM HEPES, 5 mM glucose and 1% BSA, under cold conditions (0-5˚C). The homogenate was centrifuged at 1,000 x*g* for 10 min and the precipitate thus obtained was suspended in 17.5% dextran (MW 70,000, prepared in the above buffer without BSA) and then centrifuged at 4,000 xg for 10 min. The pellet containing microvessels was suspended in 17.5% dextran and centrifuged. Microvessels were suspended in the preparatory buffer and passed through the glass bead (0.45 mm diameter) column (1.5×1.5 cm) and washed with excess volumes of buffer. The microvessels adhering to the glass beads were collected by agitating the beads in the buffer and finally suspended in 1.0 mL of HBSS at pH 7.4.

Preparation of nitrosyl-heme (Fe-NO) and Fe $[(OH)ONOO^-]^{2-}$ complex. Hemin hydrochloride (0.1%) and hydroxylamine (NH_2OH) (1%) were dissolved in NaOH (pH 10) in the presence of catalase (0.1 mg). The mixture was stirred for 1 hour and a red color was observed. Nitrosyl-heme was detected by UV diode array spectroscopy at 398 nm. Nitrosyl-heme can also be detected by luminol-chemiluminescence (luminol/H_2O_2 system) where a peak chemiluminescence within 1 second is taken. In the absence of catalase, the $ONOO^-$ formed from NO and O_2^- released during the autoxidation of NH_2OH attached to the –OH group in the Fe^{2+} of hematin to form an orange stable radical $Fe[(OH)ONOO^-]^{2-}$. The $Fe[(OH)ONOO^-]^{2-}$ complex was detected by UV diode array spectroscopy at 428 nm. The luminol-chemiluminescence response of the $Fe[(OH)ONOO^-]^{2-}$ complex was estimated by a Luminometer. $ONOO^-$ and $Fe[(OH)ONOO^-]^{2-}$ were also determined fluorometrically by dihydrorhodamine 123 oxidation to rhodamine at excitation 500 nm and emission at 528 nm (12).

UVC irradiation. The microvessels were suspended in HBSS solution at pH 7.4 and/or 9.2 and irradiated with UVC light for appropriate periods of time. They were irradiated from a distance of 10 cm, using UVC lamp (VL- 6C, 6 W -254 nm Tube, Power: 12 W, Vilber Lourmat). The supernatant HBSS (100-300 μL) was taken for chemiluminescence studies. Irradiated microvessels were homogenized on ice by sonication (1-2 min) and the resulting microvessel homogenate was used for the estimation of the membrane fluidity. Protein concentration was measured by the Bradford method (17).

Determination of NO, $ONOO^-$ and S-nitrosothiols released from skin microvessels after treatment with UVC. $ONOO^-$ was synthesized, titrated and stored as previously described (13, 14). Nitrogen radicals released from microvessels in the HBSS medium (100 μL) were estimated by luminol-enhanced chemiluminescence method in a Luminometer Berthold LB953. Luminol (10 μL) was dissolved in DMSO. Nitrosocompounds were estimated by the slow release of NO after their treatment with H_2O_2 (500 μM) to form $ONOO^-$ (14). Determination of NO was also performed as previously described (15). The supernatant (100 μL) from the microvessel suspension was mixed with 100 μL of a reagent consisting of: 20% sulphanilamide in 20% *ortho*-phosphoric acid (H_3PO_4) and 25 μM scopoletin. The sulphanilamide-scopoletin reagent (100 μL) was mixed with the incubates and the NO was monitored at room temperature (22˚C) with an excitation wavelength of 350 nm and an emission of 460 nm. The estimation of superoxide (O_2^-) released from skin microvessels after UVC irradiation was made by lucigenin-dependent chemiluminescence in the presence and absence of superoxide dismutase (SOD).

Membrane fluidity. Membrane fluidity changes in microvessels due to UVC radiation were estimated in steady-state fluorescence polarization studies of 1,4-trimethylaminophenyl-6-1,3,5, hexatriene (TMA-DPH) according to Shinitzky and Barenholz (16). Irradiated microvessels were sonicated for 2 min and the suspension (1 mg protein) was mixed with TMA-DPH (10 μL) (1 mg/10 mL cyclohexane) for 15 min. Measurements were performed in a fluorophotometer equipped with polarizers for polarization studies, at excitation of 360 nm and emission of 430 nm. The fluorescence anisotropy is expressed as $r=I_{VV}–I_{VH}/I_{VV}+ 2I_{VH}$ where I_{VV} and I_{VH} are the intensities of the emitted light oriented, parallel and perpendicularly to the plane of the exiting beam, respectively.

Results

Figures 1 and 2 show that UVC evoked a dose-dependent increase in NO and $ONOO^-$ release. The estimation of NO was made by luminol-dependent chemiluminescence in the presence of H_2O_2 (500 μM). The peak chemiluminescence was taken within 1 second and the use of PTIO (100 μM) (a scavenger of NO) allowed the verification of NO production reducing the peak by ~80% . The release of $ONOO^-$ from microvessels suspended in HBSS at pH 9.2 was estimated by the direct oxidation of luminol (10 μM) by $ONOO^-$ giving a chemiluminescence response within 1 sec. Mannitol (10 mM) a scavenger of $OH^\bullet$ radical, had no effect on the peak chemiluminescence of $ONOO^-$ while at pH 7.0 the response was reduced by 90% . The effect of UVC radiation occurred with doses of energy known to elicit the inflammatory response in intact human skin. Skin microvessels continued to release NO and $ONOO^-$ up to 60 min after a single exposure of 5-10 min to UVC radiation.

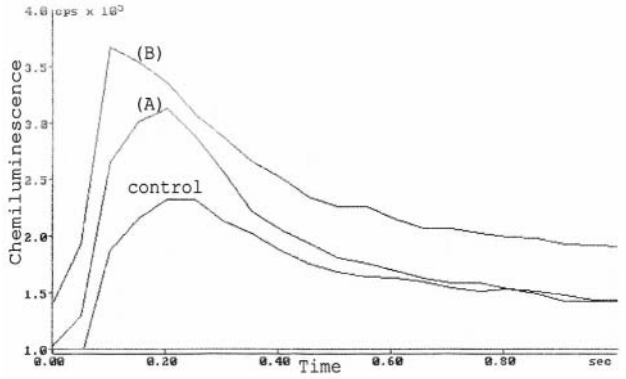

Figure 1. *Dose-dependent release of nitric oxide (NO) from UVC-irradiated microvessels isolated from rat skin. Curves (A) and (B) represent the NO production 5 min and 10 min after the irradiation, respectively.*

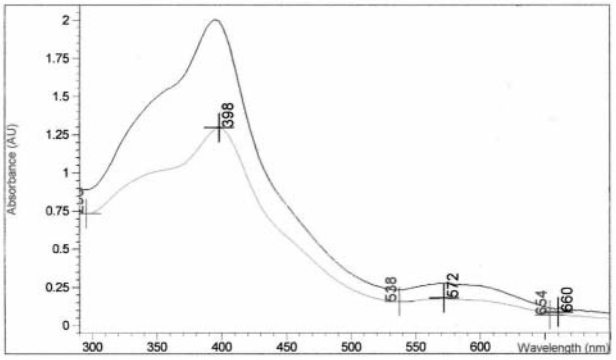

Figure 3. *Spectrum of chemically prepared nitrosyl-heme (Fe^{2+}-NO) which gives a peak at 398 nm. The accumulation of NO with time due to autoxidation of hydroxylamine (NH_2OH) in the presence of heme and catalase increased the absorbance.*

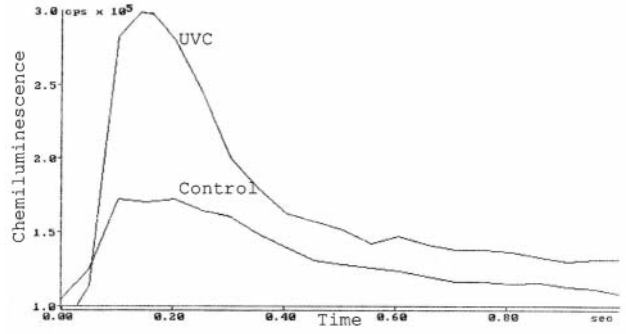

Figure 2. *Dose-dependent release of peroxynitrite ($ONOO^-$) from UVC-irradiated microvessels isolated from rat skin. Curve (UVC) shows the $ONOO^-$ production 10 min after the irradiation.*

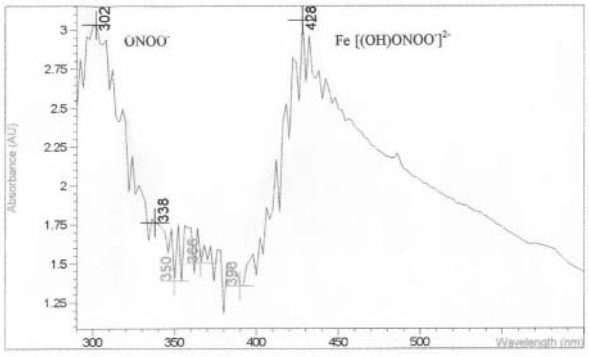

Figure 4. *Spectra of $ONOO^-$ and $Fe[(OH)(ONOO^-)]^{2-}$ which gave peaks at 302 nm and 428 nm, respectively. Both compounds were chemically formed from the autoxidation of hydroxylamine (NH_2OH) in the presence of heme (absence of catalase).*

This indicates that the initial signals that triggered NO and $ONOO^-$ release resulted in prolonged stimulation of their production.

UVC radiation of skin microvessels evoked a time-dependent increase in nitrosocompounds released in the HBSS medium as compared to non-irradiated controls. The slow release of NO from nitrosocompounds was estimated by luminol-dependent chemiluminescence in the presence of H_2O_2 as mentioned above for the estimation of NO.

The rate of NO and nitrosocompound formation by microvessels was reduced (approximately 50%) by the NO synthase inhibitor L-NMMA (1 mM). L-NMMA was added 15 min before the irradiation of microvessels to allow it to penetrate the cells and then complete its inhibitory action on NO synthase activity. Nitrosocompounds formation was also detected by the NO release in the presence of H_2O_2, an oxymyoglobin-NO assay by monitoring the loss of characteristic absorbance and an assay which detects nitrite (NO_2^-) formed from nitrosocompounds upon addition of $HgCl_2$ *via* azo dye formation from sulfanilamide and *N*-(1-naphthyl) ethylenediamine (18).

Figure 3 shows the spectrum of chemically prepared nitrosyl-heme detected with a diode array spectrophotometer at 398 nm. Arginine stabilizes nitrosyl-heme because it acts as a scavenger of superoxide released from hematin after autoxidation (19).

Figure 4 shows the spectroscopic studies of the chemically prepared $ONOO^-$ and $Fe[(OH)ONOO^-]^{2-}$ in a diode array spectrophotometer formed after the autoxidation of NH_2OH, in the absence of catalase, as described in the experimental section. $ONOO^-$ was detected at 302 nm and $Fe[(OH)ONOO^-]^{2-}$ at 428 nm at pH 9.2.

In Figure 5 (A and B) the spectrum taken from the supernatant HBSS of the irradiated microvessels 60 min after the irradiation (10 min) at pH 9.2 is shown. Figure 5A shows the spectrum of ONOO- and Fe^{2+}-NO when microvessels

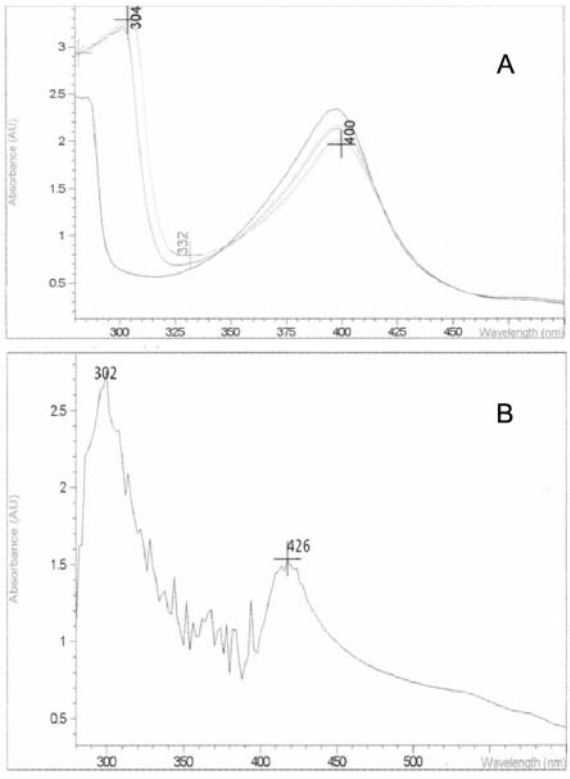

Figure 5. *A, Spectra of ONOO⁻ and nitrosyl-heme (Fe^{2+}-NO) which gave peaks at 302-304 and 398-400 nm, respectively, formed in the supernatant (HBSS pH 9.2) of microvessels in the presence of heme and catalase. The spectrum of ONOO⁻ disappeared in the presence of catalase as shown in the figure. The measurement was made 60 min after the irradiation (10 min). B, The spectra of ONOO⁻ (302-304 nm) and Fe[(OH)ONOO⁻]$^{2-}$ (426-428 nm) in the presence of heme (absence of exogenous catalase) are shown. Both spectra were formed and obtained from the supernatant (HBSS pH 9.2) of the microvessels. The measurement was made 60 min after irradiation (10 min).*

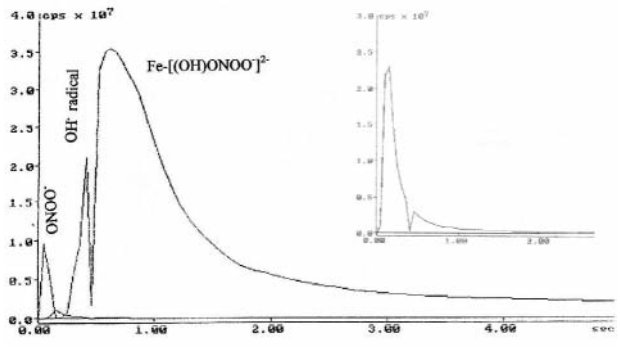

Figure 6. *Identification of ONOO⁻ and Fe-[(OH)ONOO⁻]$^{2-}$ by luminol-chemiluminescence after UVC irradiation of skin microvessels in HBSS pH 9.2, in the presence of heme (absence of exogenous catalase). The UVC-irradiated microvessels gave three peaks which corresponded to ONOO⁻ (0.25 s), OH· (0.4 s) and the complex Fe [(OH)ONOO⁻]$^{2-}$ (0.8 s).*

are irradiated in the presence of exogenous catalase. The spectra of $ONOO^-$ and Fe^{2+}-NO, which give peaks at 302-304 nm and 398-400 nm, respectively, show a slight shift with respect to the spectra of ONOO- and Fe^{2+}-NO that were chemically prepared (Figures 3 and 4). Figure 5B shows the spectrum of $ONOO^-$ and $Fe[(OH)ONOO^-]^{2-}$ in the absence of exogenous catalase.

In Figure 6, UVC-irradiated skin microvessels (1 mg protein of homogenate) in HBSS at pH 9.2, in the absence of exogenous catalase, give a luminol-chemiluminescence response with three peaks which correspond to $ONOO^-$ (0.2 s), $OH^\bullet$ (0.4 s) and $Fe[(OH)ONOO^-]^{2-}$ complex (0.8 s). This demonstrates that heme behaves as a scavenger of $ONOO^-$ and $OH^\bullet$ to form the complex $Fe[(OH)ONOO^-]^{2-}$. In the presence of mannitol (10 mM), the peak of $OH^\bullet$ is reduced by ~90% , while the $Fe[(OH)ONOO^-]^{2-}$ peak is stabilized. A similar chemiluminescence spectrum was also taken from the chemically prepared $Fe[(OH)ONOO^-]^{2-}$. The insert of Figure 6 shows the nitrosyl-heme formed and released in the HBSS medium, when microvessels were irradiated with UVC in the presence of exogenous catalase (0.1 mg/mL). The luminol-chemiluminescence response of nitrosyl-heme formed gave a peak within 1 second.

Experimental details are given in the text. This is a representative experiment which was repeated several times.

Optimal membrane function depends on the fluidity of the constituent membrane components, especially the membrane lipids. Studies on the effects of temperature changes on the fluorescence polarization (r) of diphenylhexatriene (DPH) in cell membranes showed that an increase in temperature produced concomitant diminution in the r values, which means an increase in the membrane fluidity. In the present study, the r values of TMA-DPH incorporated in skin microvessels increased in the absence of exogenous heme (1 μM) from 0.210±0.025 (controls) to 0.650±0.095, indicating a decrease in membrane fluidity consistent with the increase in the physical constraints imposed by the membrane lipid on enzymes, pumps, channels, *etc*. (20). The addition of exogenous heme restored the r value to 0.320±0.040, confirming the $OH^\bullet$ and $ONOO^-$ scavenging properties of heme.

Discussion

It was shown that skin microvessels irradiated with doses of UVC energy evoked NO and $ONOO^-$ release that was sustained for more than 60 min (Figures 1 and 2). These results indicate that UVC radiation, which is known to cause erythema and possibly cancer in human skin, stimulates NO release from skin microvasculature that in turn enhances cyclic GMP release leading to a relaxation response. The increase in NO/NO_2^- and oxygen free radicals production in

UV-irradiated skin microvessels may be an important event in determining signaling cascades and intracellular signals which can result in deranged cell differentiation (21).

In the present study, the involvement of free radicals in UVC irradiation was demonstrated by the ability of antioxidants to quench luminol chemiluminescence intensity. The chemiluminescence spectrum obtained from UVC-treated skin microvessels was enhanced as compared to non-treated control, indicating a higher oxidative stress in the treated cells which were susceptible to oxidative damage. The tumor-promoting activity of various organic peroxides and hydroperoxides has been suggested to be due to their ability to generate free radicals. The inhibitory effect of free iron chelators such as desferrioxamine and *o*-phenanthroline on such reactions indicates a role for iron in this process of oxygen free radical production and it has been postulated to require the redox cycle of an iron-centered protein which, as in the Fenton reaction, generates both peroxyl and alkoxyl radicals (22).

In addition to the generation by specific nitric-oxide (NO) synthases, NO formation from nitrite (NO_2^-) occurs in the skin. In the present study, UVC radiation activated NO-synthases and reduced NO_2^- to NO which bound to heme-centers forming the basis for NO-mediated signaling as occurs through guanylate cyclase (23). A process for the preparation of nitrosyl-heme after UVC irradiation of the skin is provided. It comprises nitrosylation of the heme iron by autoxidation of hydroxylamine (24) in the presence of catalase (Figure 3):

$$3NH_2OH \longrightarrow NO + NH_4^+ + H_2O + e^-$$

$$Fe^{3+}\ (Porphyrin) + e^- \longrightarrow Fe^{2+}\ (Porphyrin)$$

The proposed nitrosyl-heme may act as an antioxidant to retard damage induced by free radicals produced after UVC radiation. NO released from nitrosyl-heme may also act as NO donor (25). Recent data on the role of NO in diseases have stimulated efforts to pharmacologically target the NO pathway. These therapeutic strategies include NO donors that directly or indirectly release NO and agents that increase NO bioactivity. Direct NO donors include NO gas, which is useful in respiratory disorders, and the more recent classes of diazeniumdiolates, sydnonimines and *S*-nitrosothiols. Preliminary data suggest that these agents may be effective as antiatherosclerotic agents. In addition, hybrid agents that consist of an NO donor coupled with a parent anti-inflammatory drug, including nonsteroidal anti-inflammatory drugs, have demonstrated enhanced efficacy and tolerability compared with the anti-inflammatory parent drug alone in diverse experimental models (26).

NO-heme formation occurs progressively during UVC irradiation, with these complexes serving as a store of NO with concordant activation of NO signaling pathways. However, in an environment of high oxidative stress that occurs after UVC radiation of the skin, the heme which is released from hemoglobin traps the $OH^\bullet$ radical and is transformed to hematin (FeOH) (27). When hematin (FeOH) (green color) reacts with $ONOO^-$ it is converted to $Fe[OH(ONOO^-]^{2-}$ (orange color). In the present study, it was shown that this complex gives a chemiluminescence spectrum consisting of three peaks; first, the peak of $ONOO^-$, second, the peak of $OH^\bullet$ radical, and finally the peak of the complex itself (Figure 5). Therefore it is apparent that this radical complex has the potential to act as a protective and defensive mechanism against skin damage due to UVC radiation.

Numerous reports have shown that the activity and kinetics of membrane-bound enzymes and carriers can be markedly affected by the membrane lipid composition and fluidity, which confirms that the physical state of the lipids surrounding various membrane proteins, controls protein conformation and regulates protein function (23). Thus it seems clear that optimal membrane function depends on the fluidity of the constituent membrane components, especially the membrane lipids. The effects of temperature on the fluorescence polarization (r) of diphenylexatriene (DPH) in cell membranes were shown, as an increase in temperature produces concomitant diminution in the r value, which means an increase in the membrane fluidity.

In the present study, the scavenging properties of the heme, as shown by its ability to trap $OH^\bullet$ and $ONOO^-$ resulting in the formation of the complex $Fe[(OH)(ONOO^-)]^{2-}$, were demonstrated by experiments studying membrane fluidity. In preliminary experiments in microsomes isolated from UVC irradiated skin, the r value was dramatically increased suggesting a considerable decrease in membrane fluidity due to lipid peroxidation (formation of malondialdehyde) (data not shown). A similar increase of the r value was observed when microvessels were irradiated with UVC. However, in the presence of exogenous heme the r value was restored close to the levels of the non-irradiated microvessels (controls) due to the formation of the complex $Fe[(OH)(ONOO^-)]^{2-}$. Therefore, the assumption is made that the heme released from hemoglobin after UVC radiation can be incorporated into the lipid bilayer. There it can act as a scavenger of the oxygen and/or nitrogen free radicals thus preventing lipid peroxidation and any cytotoxic effect of UVC radiation on the cells. Membrane lipid bilayer fluidity has a crucial role in signal transduction for a variety of molecules which activate cellular functions, cell differentiation and proliferation. Further exploration into the mechanisms by which substances alter membrane fluidity after UVC radiation may provide clues for understanding the mechanism of tumor growth in the skin (29).

The present data suggest that heme released from hemoglobin in environments of high oxidative stress acts both as a protective and defensive mechanism by neutralizing $OH^\bullet$ and $ONOO^-$ forming the less potent radical $Fe[(OH)(ONOO^-)]^{2-}$ and as a therapeutic agent in skin microvasculature lesions by forming nitrosyl-heme that acts as a NO and possibly as nitroxyl (HNO) donor (30).

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Received September 26, 2008
Revised December 1, 2008
Accepted December 22, 2008