Vol. 175, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1991 Pages 129-133

UV Induces Oxy- and Chromanoxyl Free Radicals in Microsomes by a New Photosensitive Organic Hydroperoxide, N,N'-bis(2-Hydroperoxy-2-Methoxyethyl)-1,4,5,8-Naphtalene-Tetra-Carboxylic-Diimide

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Received January 9, 1991

Low oxygen tension, a high content of reducing equivalents and endogenous vitamin E are responsible for the resistance of cancer cells to oxidative stress-based therapy. N,N'-bis(2-hydroperoxy-2-methoxyethyl)-1,4,5,8-naphtalene-tetra-carboxylic-diimide (NP-III), capable to release radicals both in the absence and in the presence of oxygen upon UV-illumination, is a new potential anticancer agent. UV-induced reactions of NP-III in rat liver microsomes were studied under aerobic and anaerobic conditions with (i) vitamin E homologue, chromanol-α-C-6 having a shorter (6-carbon) hydrocarbon side chain and higher antioxidant activity, and (ii) the spin-trap 5,5-dimethyl-1-pyrroline-1-oxide, DMPO. UV-induced generation of chromanoxyl radicals was observed in the presence of NP-III under aerobic conditions, which was SOD+catalase sensitive. Hydroxyl-, superoxide- and alkoxyl-radical DMPO adducts were found upon UV-illumination of NP-III under aerobic conditions and only hydroxyl-radical adducts under anaerobic conditions. The light-dependent generation of oxy- and chromanoxyl free radicals and depletion of endogenous antioxidants suggests to be a promising strategy to overcome the inherent resistance of tumor cells to oxidative stress.

Oxygen radicals are well known initiators of cell damage [1]. Therapeutic effects of many anticancer drugs (e.g. anthracycline antiobiotics) are due to their ability to generate activated oxygen species [2], which oxidatively modify tumor cells but also cause side-effects in different tissues [3]. Tumor cells are more resistant to oxidative stress than normal cells, mainly due to higher concentrations of vitamin E per allylic double bond of their membrane phospholipids [4,5]. This represents one of the principle difficulties in the increase of the therapeutic efficiency of antitumor drugs with simultaneous decrease of their toxicity.

Recently photosensitizing drugs, such as haematoporphyrin derivatives [6] and psoralens [7] were introduced for the photodynamic therapy of cancer and psoriasis, respectively. Photosensitized generation of singlet oxygen and other reactive oxygen radicals is considered to be the main cause of their biological effect. Thus, damage of cancer cells by light in the presence of photosensitizing drugs is oxygen-dependent. However the oxygen tension in solid tumors is usually low which restricts the therapeutic efficiency by light-activated drugs used sofar. To overcome this limitation, a new reagent, N,N'-bis(2-hydroperoxy-2-methoxyethyl)-1,4,5,8-naphthalene-tetra-carboxylic-diimide (NP-III) containing two hydroperoxide groups and a quinoid nucleus (Fig.1) was synthesized [8]. NP-III is a compound that can generate oxy-radicals both in the absence and in the presence of oxygen by absorbing UV. Our *in vitro* studies show that NP-III was able to generate hydroxyl radicals and cause DNA strand breaks, dependent upon irradiation [8,9].

In the present investigation light-induced reactions of NP-III in rat liver microsomes were studied under aerobic and anaerobic conditions with: (i) vitamin E homologue, chromanol-α-C-

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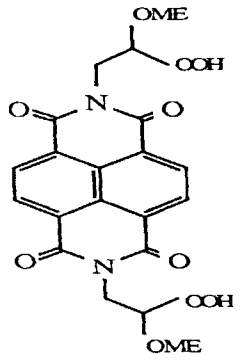

Fig. 1. Structural formula of NP-III.

6 having a shorter (6-carbon) hydrocarbon side chain, and (ii) the spin-trap 5,5-dimethyl-1-pyrroline-1-oxide, DMPO.

Materials and Methods

Membrane preparations The liver of male Wistar rats weighting 120-180 g were subjected to perfusion with 1.15% ice-cold KCl and subsequent disruption in a Potter homogenizer followed by differential centrifugation (3 000g x 10 min, 10 000g x 20 min, and 100 000g x 60 min, respectively) to obtain microsomal fractions. The concentration of protein was determined with the biuret reagent, using bovine serum albumin as a standard.

ESR measurements of chromanoxyl radicals and DMPO spin aduucts To obtain standard signals of chromanoxyl radicals from the vitamin E homologue, chromanol-α-C-6 an enzymic oxidation system (lipoxygenase + linolenic acid) was used to generate radicals in microsomal suspensions [10,12]. The incubation medium contained: microsomes (8 mg protein/ml), linolenic acid 14 mM, lipoxygenase 90 U/μl, in 0.1 M Na,K-phosphate buffer, pH 7.4 at 37° C.

ESR measurements of chromanoxyl radicals were made on a Varian E 109E spectrometer, in gas permeable Teflon tubing (0.8 mm internal diameter, 0.013 mm thickness obtained from Zeus Industrial Products, Raritan N.J. USA). The permeable tube (approximately 8 cm in length) was filled with 60 μl of a mixed sample, folded into quarters and placed in an open 3.0 mm internal diameter EPR quartz tube such that all of the sample was within the effective microwave irradiation area. Spectra were recorded at 100 mW power and 2.5 gauss modulation, and 25 gauss/minute scan time. Reaction mixture contained: 50 μl of microsomes in phosphate buffer (8 mg protein /ml), 3 μl of chromanol-α-C-6 (80 mM) and 5 μl of NP-III (0.2 mM in DMSO). ESR spectra were registered at room temperature under aerobic and anaerobic conditions by flowing oxygen or nitrogen gas through the ESR cavity. Illumination was applied directly to the ESR cavity from a UV source.

ESR measurements of DMPO spin-adducts were carried out typically as described below. To 30 μl of microsomal suspension (8 mg protein/ml of 0.1 M phosphate buffer, pH 7.4 at 37°C), were added an aliquot of NP-III (dissolved in DMSO) and 10 ml of 4.0 M DMPO aqueous solution. The reaction mixture was taken in a haematocrit capillary tube and setted in the ESR cavity. DMPO adducts were determined at 3240 magnetic field strength and the power strength of 100 mW, modulation amplitude 1.6 gauss, scan range 100 gauss, time constant 0.64.

Irradiation UVAB was provided from a solar light source (Solar Light Co.) which covers the wavelength range from 295 to 400 nm. The samples were illuminated in ESR resonator cavity (the distance between the light source and the sample was 40 cm). Total irradiation energy between 295 and 400 nm was 5 J/cm^2.

Reagents DMPO purchased from Sigma Co. Ltd. was first purified by passing through a charcol column before use. Soyabean lipoxygenase, linolenic acid, SOD and catalase were from Sigma Co. Ltd.

NP-III was synthesized from naphtalene tetracarboxylic acid and purified according to the method reported previously [8,9].

α-Tocopherol homologue with a 6-carbon side chain, chromanol-α-C-6, was a gift from Prof. R.P.Evstigneeva (Institute for Fine Chemical Technology, Moscow, USSR).

Results

Light-induced chromanoxyl radical formation was determined using chromanol-α-C-6 as the model of membrane solubilized α-tocopherol (vitamin E) [10] in the absence and presence of NP-III. Under aerobic conditions NP-III addition to microsomal suspensions did not result in any significant chromanoxyl radical signals in the dark. Upon irradiation the ESR signal of chromanoxyl radicals was observed in controls and in samples to which NP-III was added. The magnitude of chromanoxyl radical signal was increased by approximately 45 % by NP-III (Figs. 2,3). Prior addition of SOD (0.65 U/μl) and catalase (0.50 U/μl) decreased the chromanoxyl radical ESR signal by approximately 10 %. This corresponds to 70 % of NP-III-dependent fraction of the signal (Figs. 2,3).

When the light was turned off, the signal decayed within 2 min scan of the spectrum (Fig. 3). In the course of continious irradiation the intensity of the chromanoxyl radical ESR signal decreased after reaching its maximum amplitude (after about 10 min of irradiation) and the signal almost completely disappeared after 20 min (Fig. 3). Repeated irradiation after keeping the sample in the dark (5 min) did not increase any ESR signals of chromanoxyl radicals (not shown). This indicates that all the chromanol-α-C-6 was consumed due to oxidation by the light-induced radicals in the presence of NP-III. In contrast the amplitude of the light-induced chromanoxyl radical ESR signal observed in the absence of NP-III did not change significantly during at least 30 min of irradiation (not shown).

When irradiation was provided anaerobically, no significant growth of chromanoxyl radical was detected either in the absence or in the presence of NP-III. The ESR signal of chromanoxyl radicals however appeared when oxygen was admitted (Fig. 3).

The generation of oxygen radicals upon irradiation of NP-III in buffer and in microsomal suspension was determined using DMPO. Under aerobic conditions NP-III in aqueous medium did not give rise to a significant DMPO-trapped ESR signal in the dark at room temperature. However upon irradiation a large signal corresponding to the hydroxyl radical adduct, DMPO-OH, appeared (Fig. 4). Minor signals corresponding to superoxide and alkoxyl radical adducts, DMPO-OOH (O_2-) and DMPO-OR (·OR), were also observed in the ESR spectra. In the course of continious irradiation, DMPO-OOH and DMPO-OR signals decreased and the DMPO-OH signal became the major species observed. Amplitudes of all DMPO adducts signals were decreased in the presence of microsomes. The DMPO-OOH signal was especially sensitive to the presence of microsomes and almost disappeared.

Since chromanoxyl radical formation was extremely small under anaerobic as compared to aerobic conditions, the DMPO spin-adduct formation from NP-III was precisely determined under both of these conditions (Fig.5). DMPO-OH adducts were generated upon irradiation even under anaerobic conditions, although the amplitude was lower than that observed under aerobic condition (O_2 flow). DMPO-OOH and DMPO-OR adduct signals are quite small or absent under anaerobic conditions. This suggests that HO· are the primary radicals generated under unaerobic conditions.

Discussion

The present studies revealed that NP-III, a light sensitive radical releasing compound accerelates the oxidation of vitamin E homologue in membranes. The light-dependent

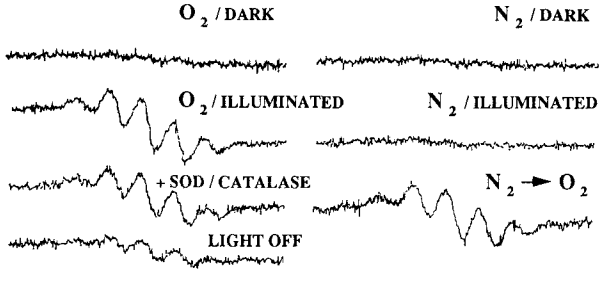

Fig. 2. ESR spectra of chromanoxyl radicals generated by UV-light in microsomal suspension incubated in the presence of NP-III under aerobic or anaerobic conditions.

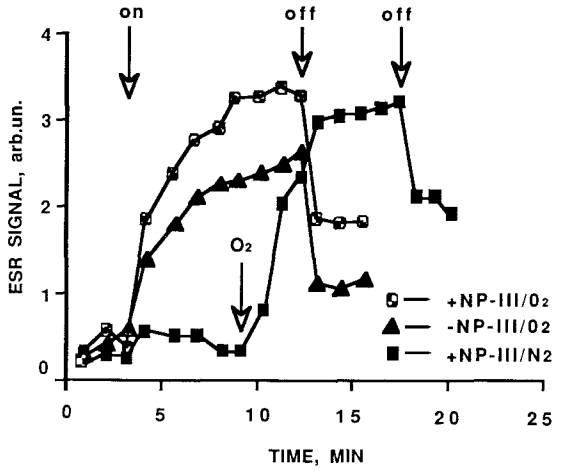

Fig.3. Time course of UV-light induced chromanoxyl radical ESR signals in microsomal suspension incubated in the presence of NP-III under aerboc or anaerobic conditions .

generation and disappearence of chromanol-α-C-6 chromanoxyl radical suggests a possible use of radical releasing drugs to reduce inherent resistance of tumor cells to oxidative stress.

The NP-III dependent chromanoxyl radical generation was light and oxygen dependent Since NP-III is decomposed by light via postulated intermediate formation of a semiquinone structure [8], the consequent generation of superoxide anion and hydroxyl radicals is possible in the presence of oxygen and this actually results in generation of DMPO-OOH and DMPO-OH adducts under aerobic conditions. UV-light may also be absorbed and may activate the flavins present in the microsomes. Thus the formation of the chromanoxyl radical may be due to oxygen radicals generated by flavin and NP-III-mediated reactions [8,11]. Generation of chromanoxyl radicals was severely inhibited under anaerobic conditions. However hydroxyl radicals were generated upon photolysis of NP-III under both aerobic and anaerobic conditions. Thus hydroxyl radicals may not be the primary species responsible for chromanoxyl radical generation.

In addition to DMPO-OH adduct, at least three other DMPO adducts DMPO-OOH, DMPO-OR and small amounts of carbon centered radicals, DMPO-R, were detected in the presence of oxygen. However both DMPO-OOH and DMPO-OR radicals were missing under anaerobic conditions. Therefore, superoxide and alkoxyl radicals are the candidates for the species

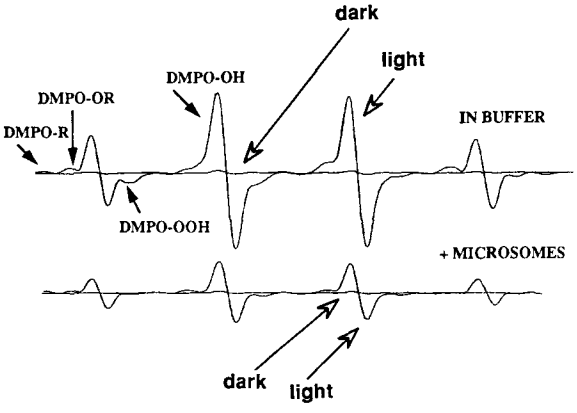

Fig.4. ESR spectra of DMPO spin adducts generated by NP-III in buffer and in microsomal suspension upon exposure to UV-light.

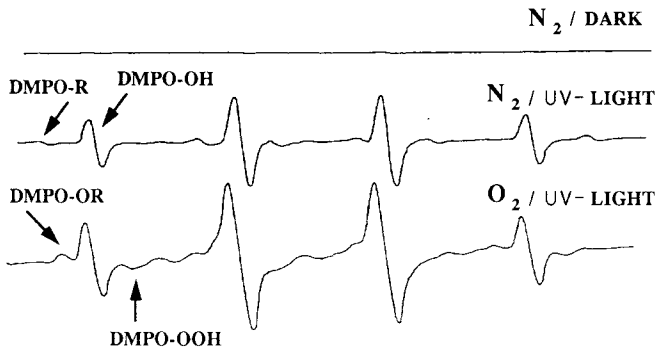

Fig.5. ESR spectra of DMPO spin adducts generated by NP-III in buffer upon exposure to UV-light under aerobic or unaerobic conditions.

responsible for chromanol-α-C-6 oxidation in membranes. Although superoxide radicals are known to react with vitamin E [13], the presence of SOD and catalase could not completely inhibit the light-induced generation of chromanoxyl radicals in the presence of NP-III. Thus alkoxyl radicals may have a potential role in the chromanoxyl radical generation. Based on our results we suggest that hydroxyl radicals do not directly react with chromanol-α-C-6 in the membrane, since chromanoxyl radicals were not detected under the same anaerobic conditions where DMPO-OH adducts were found. One possible explanation for the low reactivity of hydroxyl radicals with the vitamin E homologue, chromanol-α-C-6 might be due to their preferential location in the aqueous phase and high reactivity at the membrane surface.

Further experiments on the specificity of binding of NP-III with tumor cells and on site-specific generation of radicals are necessary to evaluate the potency of light-induced generation of different radicals by NP-III resulting in a rapid oxidation of the membrane vitamin E homologue. By means of this approach a strategy to overcome the inherent resistance of tumor cells to oxidative stress may be devised.

Acknowledgments: This research was supported by NIH grant CA 47597-03 and the National Foundation for Cancer Research.

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