EPR Study on the Role of Riboflavin Used in Photo-Oxidative Collagen Cross-Linking

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This paper presents an EPR study on the free radicals formed by exposing riboflavin to light in the presence and absence of collagen. DMPO and PBN have been used as spin-traps for the short-lived free radicals formed during this process. The results showed that mainly hydroxyl radicals are trapped, as the corresponding spin-adducts of DMPO and PBN were identified by EPR spectroscopy; in the presence of collagen, these are adsorbed on the collagen fibers.

Keywords: riboflavin, spin trapping, collagen cross linking

Flavins are responsible for light induction of free reactions in cell medium, and this complex process, mediating interactions between light and tissue has been previously studied extensively [1-4]. Among them, riboflavin (vitamin B2, fig. 1) represents one of the most known flavin coenzyme.

Collagen represents the major structural protein in vertebrates, being predominantly located in the extracellular matrix. This protein is characterized by a triplet helical structure with unusual strength and stability [5] due to various interactions: van der Waals, electrostatic, dipole-dipole, etc. Three triple helical polypeptides form a rope-like right-handed supercoil with a length of approximately 300 nm and a diameter of 1.5 nm. These supercoils are stabilized by hydrogen bonds between glycine and proline amino acids located in neighboring chains, and by an extensive water network which can form hydrogen bonds between several carbonyl and hydroxyl peptide residues. The helical part is flanked by short non-helical domains named telopeptides, which are responsible for fibril formation and natural cross-linking process. The fibrils organize into fibers which further can form large fiber bundles. Fibers and fiber bundles are stabilized by intermolecular cross-links [5, 6]. Collagen is present also in the eye structure, determining the biochemical characteristic of cornea. Keratoconus – disease which is characterized by weakness of cornea and biochemical alterations of cornea and some studies have shown that this disease is accompanied by a modified configuration of stromal collagen lamella [7, 8].

Keratoconus is a non curable disease, but one way to prevent or stop its progression consists in applying a topical dose of riboflavin drops to the cornea and exposing the cornea to a low amount of ultraviolet A (UVA) light [9]. Keratoconous can develops due to the fact that some people are unable to produce certain enzymes, which can prevent accumulation of the free radicals and in this way determine corneal thinning and weakening. A photosensitizing substance such riboflavin in the presence of UVA enhances corneal strength and integrity by increasing collagen cross-linking [10, 11]. Due to riboflavin, reactive oxygen species are formed under light, and these

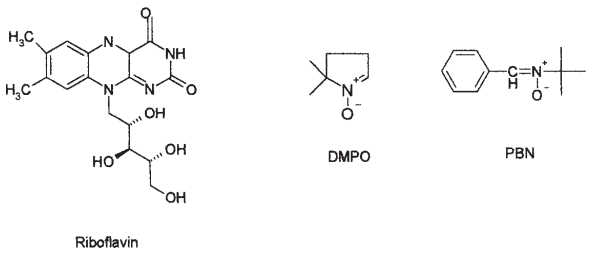

Fig. 1. Chemical structures of riboflavin (vitamin B2), DMPO and PBN

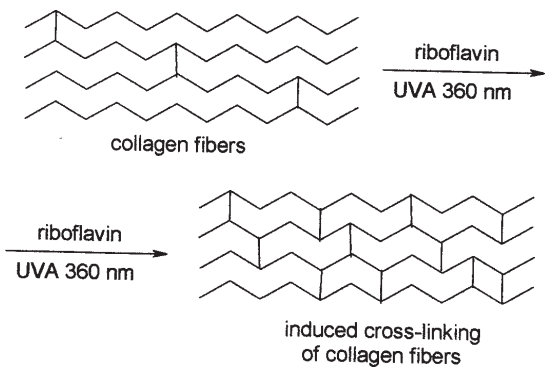

Fig. 2. Schematic representation of the cross-linking process

induce a polymerization type process (cross-linking) on collagen fibers. A schematic representation of processes which take place during corneal collagen cross-linking is suggested in figure 2.

This method, corneal collagen cross-linking, used to stop the evolution of keratoconus was implemented in 90's in Dresda, and now it is applied in other European countries. In our country, at best of our knowledge it is applied in some clinics in Bucharest and Cluj.

In this study we used electron paramagnetic resonance (EPR) spectroscopy to detect free oxygen-radicals formed during illumination (light exposure) of systems containing riboflavin (vitamin B2), or in the presence of other compounds able to generate radicals, in order to understand the role of riboflavin as radical generator (or

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mediating radical generator) or as radical scavenging. As free radicals generated in such systems are reactive, their formation was evidenced by formation of adducts corresponding to spin traps such DMPO and PBN.

Experimental part

Materials

Riboflavin was purchased from Carl Roth GmbH, collagen from Fluka (from bovine Achilles tendon, lyophilized form), *N*-tert-butyl-α-phenyl-nitrone (PBN), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and sodium ascorbate from Aldrich, sodium sulphite from Chimopar. Double distilled water was used throughout the experiments. A hand-held UVA 8W lamp has been also used.

Methods

EPR spectra were performed at room temperature on a Jeol FA100 EPR spectrometer. The following parameters were used: center field 3358 G, sweep width 80 G, microwave power 1 mW, modulation amplitude 0.3 G, amplitude between 700-1000, microwave frequency 9.43 GHz, sweep time 60 s and time constant 0.1 s. The spectra simulation was carried out using WinSim free software.

Sample preparation

Stock solution of DMPO and PBN in water at a concentration of 0.1 M were prepared and added to the solutions containing riboflavin (0.1%). For the EPR measurements the mixture was then loaded into capillary glass tubes (inner diameter 1 mm).

Results and discussion

Several types of experiments have been carried out. For the beginning, in all cases blank samples have been tested to avoid fake results; thus, samples containing only DMPO and PBN were exposed to similar conditions (e.g chemicals, compounds, light) and as a result no EPR signals were evidenced initially.

The next step consisted in the exposure of riboflavin aqueous solution to day light in the presence of DMPO or PBN, as spin-traps for the short-lived radicals formed by light-induction enhanced by riboflavin. In the case of DMPO, an EPR spectrum was obtained, consisting in two components – one corresponding to DMPO adducts of HO· radical (a four line spectrum), and the other corresponding to a decomposition product of DMPO (a three line spectrum) (fig. 3a). The EPR parameters of the adduct DMPO-HO· were a_N = 15.04 G and a_H = 14.73 G, as literature data showed [12]. Using PBN as spin trap for the riboflavin solution exposed to day light no spin adduct was observed; the result may be explained by a lower capacity of PBN to capture oxygen centered radicals.

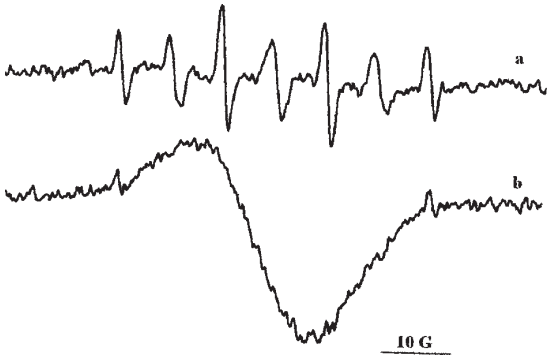

Fig. 3. (a) EPR spectra of DMPO-OH adduct formed from riboflavin solution exposed to day light, and (b) radical adduct adsorbed on collagen fibers

In the experiments involving the presence of collagen (besides riboflavin and a spin-trap), the mixture solution has been exposed to day light for few hours (collagen was suspended in riboflavin solution). We have chosen this non-selective method of irradiation of the systems containing riboflavin taking into account that ambient light also consists of a radiation in the ultraviolet (UVA 400-320 nm, UVB 320-260 nm) [13]. The recorded EPR signal consisted in a very broad line (fig. 3b), which usually is due to the situation in which radicals are adsorbed or immobilized on a surface [14, 15]. We have performed also experiments in which we used UVA irradiation at 360 nm generated by a lamp; after half an hour, no adducts were evidenced in this case and this result is presumably due to the low intensity of UV light emitted from our lamp.

In order to explain the broad line spectrum observed for the system containing collagen, the solid phase (collagen) was separated from riboflavin solution; subsequently, EPR spectra were recorded for each phase, immediately after exposure and after 24 h. The solutions separated, containing riboflavin, didn't show any EPR signal for the case in which DMPO was the spin trap. Both in the case of using DMPO and PBN as spin trap were evidenced EPR signals on wet collagen. As we showed previously, in the case of DMPO the spectra shown a broad line, corresponding to a situation in which radicals are adsorbed on the collagen surface and interact strongly (fig. 3b). This result proves the interaction between radical reactive species with collagen fibers.

For PBN, the adduct adsorbed on collagen fibers after exposure to the day light was not observed immediately, but was observed after one day, also as a radical adsorbed on collagen fibers (fig. 4a). Solution was showing also an EPR signal of three lines, which may be a result of PBN adduct degradation (fig. 4b). Because collagen is not completely dry and may contain also solution adsorbed, EPR spectra attributed to PBN adduct adsorbed on collagen fibers is a result of two components – adduct spectrum and the oxidized or decomposition form of PBN from liquid. EPR parameters for spectra of PBN adsorbed on collagen were the following: a_N = 14.49 G and a_H = 2.33 G.

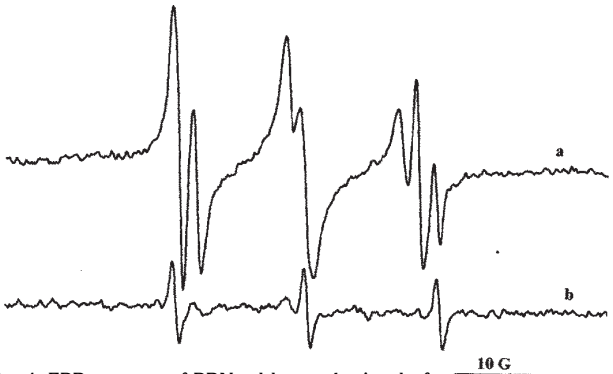

Fig. 4. EPR spectra of PBN adducts obtained after exposure to sun light and left overnight on the system containing riboflavin and collagen a) PBN adducts adsorbed on collagen fibers; b) oxidized or decomposition form of PBN adduct in solution

To check out if indeed riboflavin has a pro-oxidative effect in such systems, we combined the same system with a reducing compound, as ascorbic acid or sodium sulphite. Our experiments showed that using ascorbic acid as antioxidant, no hydroxyl radical has been noticed, but the formation of the ascorbic radical has been recorded (fig. 5a). The highest intensity has been recorded just after mixing all components, and the intensity of the EPR signal decreases in time (fig. 5b).

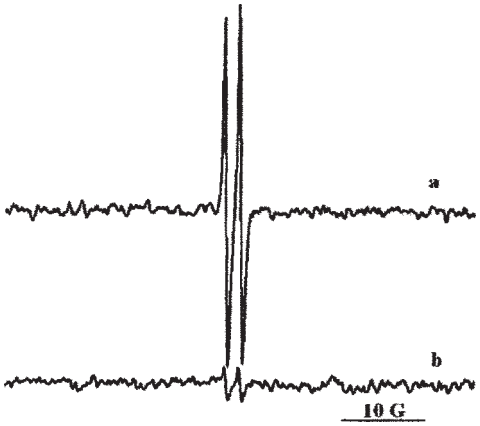

Fig. 5. EPR spectra of ascorbic radical (a) – initial, b) after 20 min

The aqueous solution containing Na_2SO_3 (as a reducing agent) and riboflavin, in the absence of spin trap, is EPR silent. In the presence of spin-traps (DMPO or PBN), formation of free radicals was evidenced. In figure 6a-d are presented the EPR spectra of DMPO adducts recorded at different time interval after solution preparation, and thus can be observed the decay of the signals. The main spin-adduct radicals observed initially (fig. 6a) is due to the formation of the sulphite radical (probably from oxidation of the sulphite anion by hydroxyl radical, a very powerful oxidant). Hyperfine coupling constants for DMPO-.SO3 adduct (a_N = 14.58 G, a_H = 15. 92 G) obtained by simulation of EPR spectra from fig 6a, are consistent with the literature data [12]. In time, the EPR spectrum is changed to the well known HO-DMPO spin-adduct (fig. 6d). For the case of PBN spin-trap, the same trend has been noticed; thus, a strong signal was recorded at the beginning and following decay in time.

The reactive radicals generated by interaction with light of riboflavin interact with reducing agent, fact evidenced by changes in EPR spectra of adducts detected.

Conclusions

The result reported here shown that aqueous solution of riboflavin under light conditions generates short-lived radicals, mainly identified as hydroxyl radicals, as trapped by DMPO or PBN spin-traps and evidenced by EPR spectroscopy. The reactive radicals are adsorbed on collagen fibers, facilitating in this way a subsequent process such cross linking. In the presence of a reducing agent, adducts corresponding to oxygen centered free radicals are evidenced in delay.

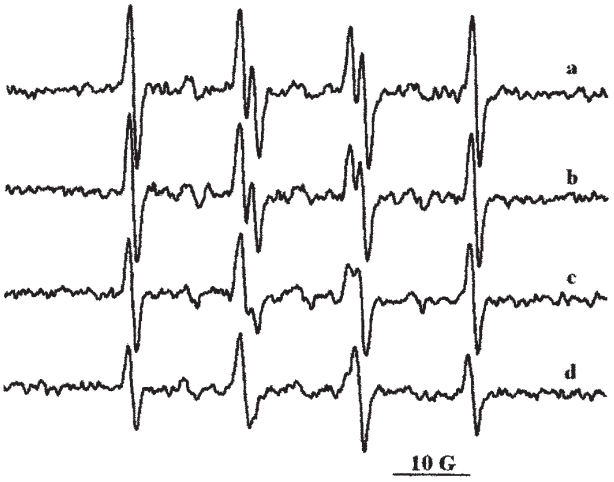

Fig. 6. (a) EPR spectra of DMPO adducts from fresh solution of Na_2SO_3 and riboflavin, (b) after 2 min, (c) after 6 min, (d) after 17 min

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