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Dihydroneopterin as a scavenger of nitrogen centered radicals

Karl Oettl, Joachim Greilberger and Gilbert Reibnegger

Institute for Medical Chemistry and Pregl-Laboratory, Karl-Franzens-University Graz, Harrachgasse 21II, A-8010 Graz, Austria

Running title: Dihydroneopterin and nitrogen radicals

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Summary

Dihydroneopterin has recently been shown to either promote or decrease formation of free radicals or radical-mediated reactions, depending on the conditions. We report here the scavenging activity of dihydroneopterin on nitrogen centered radicals. Diphenylpicrylhydrazyl (DPPH) and the radical cation of 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) were used. Dihydroneopterin showed scavenging properties against either compound. In the case of DPPH radical scavenging by dihydroneopterin was comparable with trolox. In the ABTS system, dihydroneopterin had an even better capacity of radical scavenging as compared to trolox.

Introduction

A number of papers published during the past decade reported the interference of pterins with free radical reactions (1-17), like chemiluminescence of luminol (1, 2, 4, 5, 9, 10), LDL-oxidation (6), apoptosis (8), toxicity of hydrogen peroxide and chloramine T against bacteria (3, 11), nitration of tyrosine (14) or formation of reactive oxygen species (15-17). For both, radical scavenging and radical promoting activities, most studies were focussed on oxygen centered radicals. However, the reactivities of antioxidants with different radical species may vary considerably (18).

For a complete understanding of an *in vivo* situation concerning pterins and free radicals reactive nitrogen species have to be considered, too. For example, recently the interference of pterins with nitration of tyrosine by peroxynitrite has been reported (14). Moreover, nitrogen centered radicals are often used for the characterization of the antioxidant properties of compounds and biological fluids (19-24). We therefore tested the radical scavenging activity of dihydroneopterin with two systems frequently used for the measurements of antioxidative properties. Both assays are based on nitrogen-centered radicals and in

both assays trolox, a water soluble form of vitamin E, was used as a standard antioxidant.

Material and Methods

Reagents

7,8-Dihydroneopterin was obtained from Dr. Schircks laboratory (Jona, Switzerland). Diphenylpicrylhydrazyl (DPPH), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and tris (tris-(hydroxymethyl)-aminomethane) were from Sigma (Vienna, Austria). All other reagents were obtained from E. Merck (Darmstadt, Germany).

Scavenging of DPPH:

The use of DPPH for the determination of antioxidants was first described by Blois (25). An ethanolic solution of DPPH (100 μ l, 300 μ M) was mixed with increasing concentrations of dihydroneopterin and trolox, respectively, in 10 mM Tris buffer, pH 7.4. The final solution had an ethanol concentration of 50 %. After 20 minutes at room temperature the absorbance at 517 nm was measured against 50 % ethanol in Tris buffer as a blank (26).

Correspondence to: Dr.Karl Oettl, Institute of Medical Chemistry and Pregl-Laboratory,
Harrachgasse 21/II, A-8010 Graz, Austria
tel: ++443 316 380 7544, fax: ++43 316 380 9610, e-mail: karl.oettl@kfunigraz.ac.at

Scavenging of 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS)

A commercial testkit, Radox Total Antioxidant Status, (Dr. Franz Tatzber KEG, Klosterneuburg, Austria) was used. The assay is based on the formation of the radical cation ABTS⁺, and its scavenging by antioxidants (19). The solutions of the testkit were prepared according to the instructions and contained chromogen, standard and substrate. The chromogen solution consisted of 6.1 μ M metmyoglobin and 610 μ M ABTS, substrate was 250 μ M hydrogen peroxide and as standard a solution of 1.6 mM trolox was used. The assay was not performed as a single time point but as a kinetic measurement and the lag time of the onset of radical formation was calculated (19, 27). For the assay 10 μ l of sample were mixed with 250 μ l chromogen solution in a cavity of a 96 well plate. To the mixture 50 μ l of substrate were added and the measurement of the absorbance at 620 nm was started immediately. From the intersection of the kinetic curves of blank (water) and standard the start time of the assay was calculated using a Microsoft Excel program. By this procedure different handling times of different plates are corrected. The lag times of samples were obtained by the same procedure and were compared to the values obtained using trolox. Two sets of experiments were performed in triplicate.

Results

The scavenging of DPPH radical by dihydroneopterin and trolox is shown in Fig. 1.

Both, dihydroneopterin and trolox gave a concentration-dependent decolorization of DPPH. The behaviour of dihydroneopterin is essentially the same, compared with trolox. In the experiment shown in the upper panel 50 μ M DPPH were used, and 100 μ M in the lower panel. In both cases maximum decolorization is achieved at about half the concentration of the antioxidant compared to DPPH. This is in accordance with earlier reports describing the antioxidant activity of trolox (26).

Using the ABTS assay we found a pronounced lag phase in radical formation when trolox was added to the assay mixture (Fig. 2, upper panel). After the lag phase radical production starts with the same rate as in the blank. When dihydroneopterin was used there was again a concentration-dependent lag phase followed by a radical formation rate as in the blank. The concentration dependence of the lag phase is shown in Fig. 2, lower panel showing straight lines for both trolox and dihydroneopterin.

Fig. 1: Scavenging of DPPH radicals. DPPH (50 μ M, upper panel and 100 μ M, lower panel) was mixed

with increasing concentrations of dihydroneopterin (●) or trolox (○) in Tris buffer containing 50 % ethanol. After 20 min the absorbance at 517 nm was measured. The results of a typical experiment are shown.

Discussion

Evidence is growing that pterins are involved in radical mediated reactions (1-17). However, a reliable view of the *in vivo* role of pterins concerning free radicals is hardly available. Although fully and partly reduced pterins have been described to be radical scavengers, these compounds are reducing agents and like ascorbate may act also prooxidatively in the presence of transition metal ions (15-17).

In this work we used two different nitrogen centered radicals, and we tested the ability of dihydroneopterin to scavenge these radicals. Both compounds are decolorized upon uptake of electrons and hence decolorization reflects the electron donating ability of the antioxidant used (20, 21, 23, 25). The concentration dependent scavenging of DPPH is essentially the same using dihydroneopterin and trolox, respectively. Both show maximum scavenging at approximately half the concentration of DPPH in

the assay mixture. This means that both compounds donate two electrons per molecule. However, the reaction may proceed via different mechanisms using different solvents (24). In the ABTS assay we found the same rate of radical formation in the blank and in the mixture containing dihydroneopterin after the lag phase indicating that dihydroneopterin is not interfering with the radical generating system. This observation is important as radical

Fig. 2: Scavenging of ABTS radicals. Radical formation was achieved by the ABTS system in the absence of antioxidants (○), in the presence of 26 μ M trolox (◊) and in the presence of 8.2 μ M dihydroneopterin (●), respectively. Radical formation was followed by the absorbance at 620 nm. Antioxidant activity is indicated by a lag phase in the formation of radicals (upper panel). In the lower panel the concentration dependence of the lag time for trolox (◊) and dihydroneopterin (●) is shown.

formation is catalyzed by metmyoglobin and interferences of dihydroneopterin with metal-dependent radical formation has been demonstrated (18, 28). On the other hand the chelating ion cyanide

was found to poison the ABTS system without scavenging the formed radical (27). Similar to trolox the lag phase depends on the concentration of dihydroneopterin. However, the slope in the plot of lag time vs. concentration (Fig. 2, lower panel) is 50 % higher for dihydroneopterin (0.21) compared to trolox (0.14). This means if trolox donates two electrons dihydroneopterin donates three electrons to the ABTS⁺ radical. The additional electron is not a contribution of the hydroxyl groups in the side chain of dihydroneopterin as neopterin has completely no effect on radical formation and the preformed radical in the ABTS system (data not shown).

From our data we cannot explain the differences in mechanism of decolorization of the ABTS⁺ radical between dihydroneopterin and trolox. However, it is clearly demonstrated that in the used radical systems dihydroneopterin acts as an electron donating radical scavenger. The same property might be responsible for the prooxidative characteristics of dihydroneopterin in the presence of metal ions. We therefore speculate that the activity of dihydroneopterin concerning radical scavenging or promoting in a physiological environment similar to ascorbic acid depends strongly on the presence of metal ions as.

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