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Abstract

Understanding the role of oxidative stress in brain as well as developing medical strategies to reduce its damaging potential in the aging process and pathogenesis of cancer, neurological diseases like Alzheimer's diseases and Parkinson's diseases and other incurable illnesses is an important direction in medicine and biochemistry over the world. This review outlines the processes by which hROS may be formed, their damaging potential and determinations methods. Also, the questions upon the nature of reactive hROS in a Fenton (like) system plays a crucial role will be addressed on this part and several lines of evidences will be presented in order to clarify this issue. Highly reactive hydroxyl radicals (hROS) have been implicated in the etiology of many diseases, therefore monitoring of hROS should be extremely helpful to further investigate and understand the role of hROS in the pathogenesis of neurological disorders and to develop medical strategies to reduce the damaging potential of hROS. The very short half-life of OH• requires the use of trapping agents such as salicylic acid or phenylalanine for detection, but their hydroxylated derivatives are either unstable, or implicated as reactant in biochemical processes. Based on already successfully in vitro and in vivo work done in our group in the past two decades, we decided to use sodium terephthalic acid as a trapping agent, the hydroxylation of which yields only one stable and highly fluorescent isomer, 2-hydroxyterephthalate (OH-TA).

Introduction

As reported by Sies and Cadenas [1], oxidative stress is defined as an imbalance between oxidants and antioxidants in favor of oxidants leading to a disruption of redox signaling and its control or to molecular damage. As established in various reported studies, the brain appears to be most susceptible to this damage. Due to the presence of high amounts polyunsaturated of fatty acids, low levels of antioxidants such as glutathione or vitamin E, and the elevated content of iron in specific areas, as for example the substanitia nigra and globus pallidus, the ability of brain to bear oxidative stress is limited [2]. Several recent reports are in support of the hypothesis that highly hydroxyl radicals are the major species responsible for oxygen toxicity in biological processes [3]. One possible way in which ROS are transformed into hROS is the Fenton Reaction (where hydrogen peroxide is reduced at the iron center with generation of free hydroxyl radical). Haber and Weiss suggested that the highly reactive hydroxyl radical (OH•) could be generated by an interaction between superoxide ($\bullet O^{2-}$) and hydrogen peroxide (H_2O_2) [4]:

•O2- + H2O2 → OH• + OH

Thermodynamically in living cells this reaction system requires some sorts of catalyst to proceed [5]. Although many transition metal ions such as Fe^{2+}, Cu^{2+}, Ti^{3+}, Co^{2+}, Cr^{2+} are capable of catalyzing this reaction [6], in this context iron is the most important transition metal, because of its high abundance on mammalian cells. Copper is probably more important than iron for oxidative damage to DNA [7]. The iron-catalyzed Haber– Weiss reaction, which makes use of Fenton chemistry, is now considered to be the major mechanism by which the highly reactive hydroxyl radical is generated in biological systems [8]. The nature of the oxidizing species produced via a Fenton system strongly depends on the chemical environment, like the pH, the ligand(s) of the Fe (II) complex or the presence or absence of O2 [9, 10, 11]. In vivo only iron and copper are present in high enough concentration to be relevant in Haber Weiss reaction [12]

Fe3+ + •O2- → Fe^{2+} + O_2

Fe^{2+} + H_2O_2 → Fe^{3+} + OH• + OH-

Reactive oxygen species (ROS) is a collective term that covers several substances, ranging from the relatively unreactive H_2O_2 through O^{2-} and singlet O_2 to the highly reactive oxygen species (hROS), which may exist as free hydroxyl radicals (OH•), as bound (''crypto'') radicals or Fe(IV)-oxo species. In addition, each species of ROS has a characteristic chemical reactivity [13], for example, $^1O_2$ reacts with anthracenes to yield endoperoxides in the Diels-Alder mode [14], whereas OH• can react directly with aromatic rings to yield hydroxylated products [15], while NO reacts with guanine to yield the deaminated compound [16]. In order to fully understand the biological roles of each ROS spacies is necessary to develop highly selective and sensitive methods. Furthermore, hROS can be generated from numerous sources in vivo. Mitochondria could be one of the most important in vivo sources of hROS generation, also superoxide anion and hydrogen peroxide can be generated from this organelle. The quantity produced under both normal and pathologic conditions is unknown [17-22]. The number of different enzymes have been shown to be capable of generating hROS is extensive, and includes the cytochromes P450, various oxidases, peroxidases, lipoxygenases and dehydrogenases [5]. In addition, NADPH oxidase is well known to generate hROS as part of its antibacterial function on phagocytic cells, this enzyme also appears to be present on numerous other cells and may have important signal transduction activities [22]. There exists convincing evidence that reactive oxygen species (ROS), including O^{2-} H_2O_2, OH• , and NO•, play an important role in numerous pathologies including neurodegenerative diseases, ischemic or traumatic brain injuries, cancer, diabetes, liver injury, and AIDS [23-30]. Reactive hydrogen species and singlet oxygen have been shown to hydroxylate purine residues in DNA, forming the 8-oxo-7, 8-dihydro-20-deoxy-derivatives of adenosine and, oxidized purine or pyrimidines can cause functional problem [31-33]. The free radical ageing theory (which hypothesizes that increases in ROS accompany aging, leading to functional alterations, pathological conditions, and even death), proposed by Harman, postulates that damage to cellular macromolecules via free radical production in aerobic organisms is a major determinant of life span [34,35].

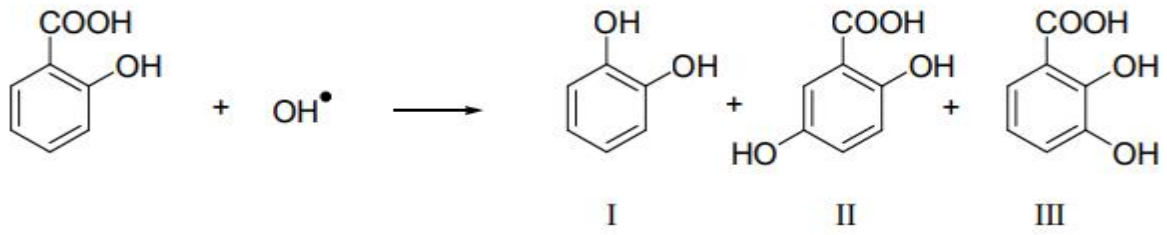
Methods for hROS detection

Direct measurement of the hydroxyl radical is practically impossible due to its very short lifetime around 1 ns and extreme reactivity. The only one direct method of detection hydroxyl radicals is electron spin

resonance including spin trapping technique. The spin trap coupled to electron spin resonance has not been proved to be a good method for measurements of hydroxyl radicals because of poor sensitivity, instability of the spin trap adducts [36, 37]. Moreover, its application to in vivo experiments, in particular in freely moving animals, is unpractical because of technical difficulties, which include high disturbing noise levels and quenching in vivo [38]. Therefore, in order to fully understand the biological roles of hydroxyl radicals, a considerable amount of research has been conducted on the developing highly selective and sensitive analytical methods to monitor hydroxyl radicals in biosystems. To address this issue several indirect methods using different chemical traps based on hydroxylation of aromatic compounds have been suggested and are widely used for monitoring formation of hydroxyl radicals in biological systems [39].

Salicylate (SA)

Salicylic acid has been mainly used in the past two decades in both in vitro and in vivo settings as the trap to determine OH• by measuring its hydroxylation products, (scheme 1.1)

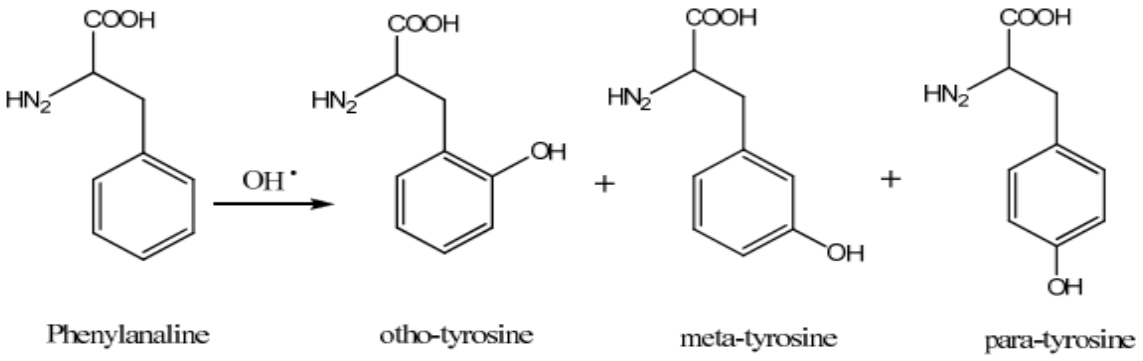


Scheme 1.1. *The Hydroxylation of Salicylic acid.*

Although widely used, this method has several fundamental disadvantages, hydroxylation of salicylic acid provides two mayor hydroxylated products 2, 3-dihydroxy salicylate (49%) and 2, 5-dihydroxy salicylate (40%) accompanied by cathecol which is formed only in small amount (11%) [39]. Because 2,5-DHBA can also be produced enzymatically by the cytochrome P450 [40, 41], only 2,3-DHBA can be used for hROS determination. Moreover, Salicylic acid is also found to affects inflammatory responses through inhibition of cyclooxygenase [42, 43, 44, 45], which is itself an important source of ROS and the means by which prostaglandins are produced [46]. Furthermore, salicylate inhibits phospholipase C and interacts with several transition metal ions [47].

Phenylalanine

Hydroxylation of phenylalanine (scheme 1.2) has been suggested as an alternative assay for hydroxyl radical determination [48],

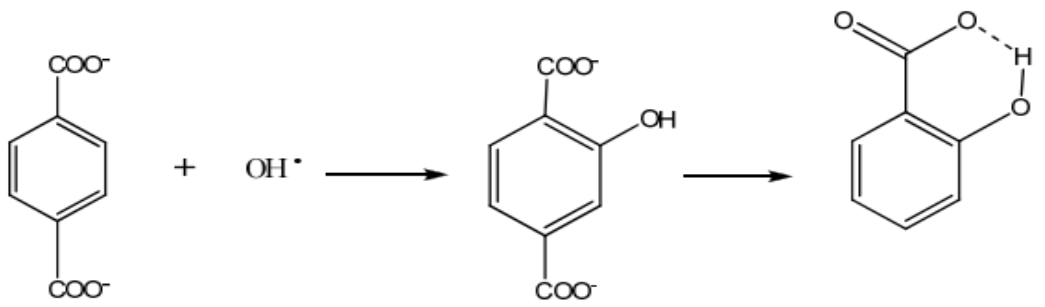


Scheme 1.2. *The Hydroxylation of phenylalanine*

Phenylalanine reacts with hROS to produce meta-, para-, and ortho-tyrosine, and paratyrosine is an endogenously product, which is formed from L-phenylalanine by the enzyme phenylalanine hydroxylase (EC 1.14.16.1) and therefore cannot be used for detection of hydroxyl radicals. Kinetic studies have shown that hydroxylation of phenylalanine is rather slow [41, 48] and the amount of hROS required to overcome the detection limit, about 50 nM, is rarely present in tissues, even in abnormal conditions [41, 49]. All these considerations raise serious questions about the validity of in vivo results obtained using those methods.

Terephthalic acid (TA)

Compared to either salicylic acid or phenylalanine, TA^{2-} (scheme 1.3) has properties that make it a superior trapping agent for OH• determination. Neither TA^{2-} nor OH-TA are normally present in tissues, or react in biochemical processes, and no toxicity has been reported regarding the parent compound [41]. In principle, the high sensitivity and reactivity of TA^{2-} with hROS allows relatively low concentrations of TA^{2-} to be used for reliable detection of hROS formation under in vivo conditions [51]. The detection limit of OH-TA in vitro was estimated to be equivalent to 0,5 nM [52]. The presence of TA^{2-} in the perfusion fluid did not affect basal or evoked release of the amino acid neurotransmitters aspartate, glutamate, taurine and GABA [51, 59]. The hydroxylated product is very stable and could allow continuous near real-time monitoring of hROS directly in the extracellular fluid of the neostriatum of freely moving animals, by coupling the microdialysis probe outlet tubing to the analytical system. It was shown that o-phthalaldehyde (OPA) does not affect OH-TA fluorescence when it was used for derivatizing amino acids. Therefore, determination of the release of amino acid neurotransmitters and hROS formation can be performed within the same microdialysis experiment.



Scheme 1.3. The hydroxylation of terephthalic acid due to radical oxidation.

4-Hydroxybenzoic acid (4-HBA)

Detection of hROS with 4-hydroxybenzoic acid (4-HBA) is less complicated, because only one hydroxylation product, 3,4-dihydroxybenzoic acid (3,4-DHBA), is formed in significant amounts (see Fig. 3). Thus, from a chemical viewpoint, this method should provide a much more reliable possibility for hROS quantitation. It has been used in microdialysis experiments, using HPLC separation with electrochemical detection [66, 67, 68]. Although 4-HBA can be hydroxylated by monooxygenases from some microorganisms, this apparently does not occur in mammals, suggesting its suitability as a chemical trap for hROS determination in vivo. As 4-HBA is an endogenous compound that shows little or no apparent toxicity, it has been claimed that it could be also used for human studies [68].

However, it is necessary to use high (mM range) concentrations of 4-HBA and further work is necessary to assess whether any biochemical processes are affected by 4-HBA, or its metabolites, at such levels, particularly in view of it being a substrate for 4-hydroxybenzoate polyprenyltransferase (EC 2.5.1.39), which is involved in the biosynthesis of ubiquinone [68].

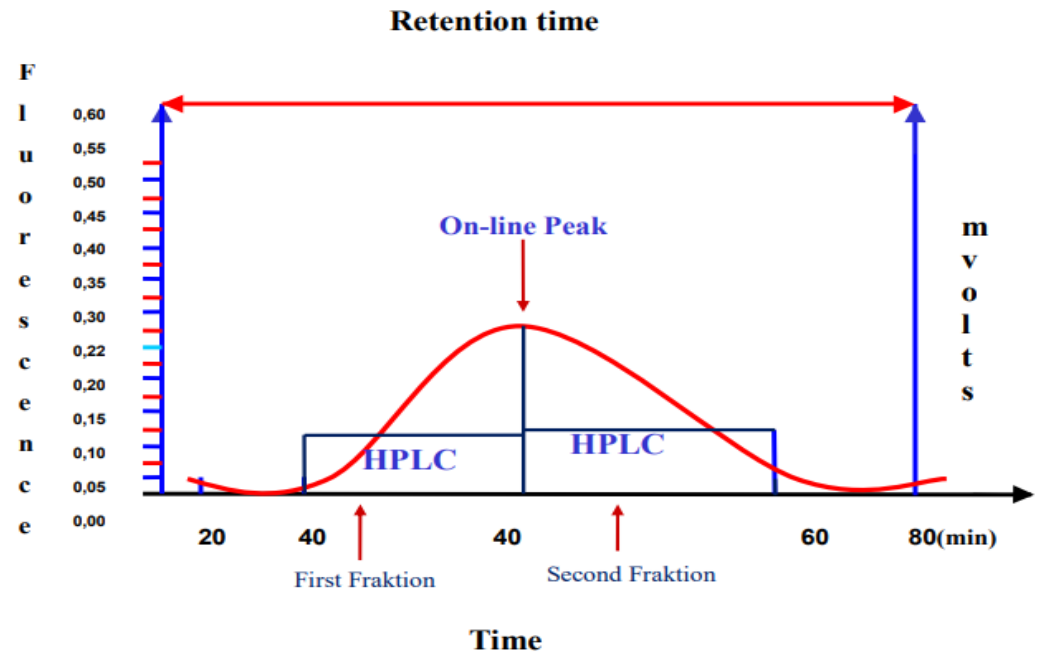
In vivo electron-spin resonance (ESR)

Electron spin resonance, also known as electron paramagnetic resonance (EPR) spectroscopy, can be used to detect molecules with unpaired electron spin states, present in radicals, transition metal complexes, like ferryl species, or molecular oxygen. However, the sensitivity is relatively low and high disturbing noise levels have limited its use in vivo. Short-lived species, like hROS, cannot directly be detected but may be transformed to more stable radicals with so-called spin traps. Among the most widely used substances for that purpose are 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO), a-phenyl-N-tert-butylnitrone (PBN) or a-(4-pyridyl-1-oxide)- N-t-butylnitrone (POBN). As spin traps react specific with reactive oxygen (ROS) or nitrogen (RNS) species.

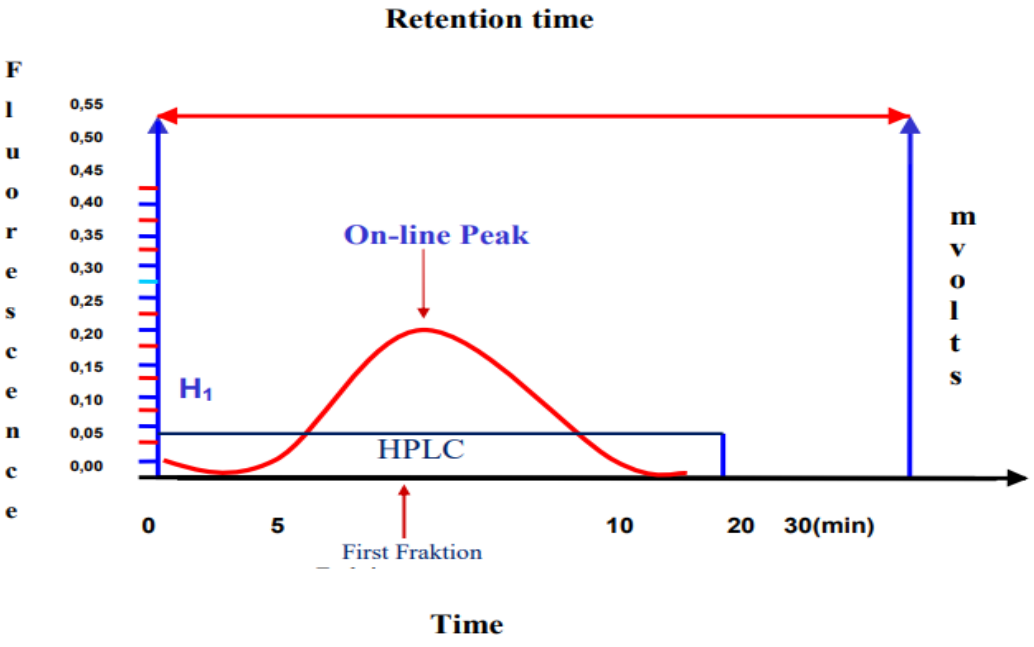
HPLC and online determination, comparison of two methods

The integration of the fluorescence detection with the capillary tube cell coupled to the in vivo microdialysis probe to form an on line electro analytical system for continuous monitoring of hROS in brain dialysate of rats is presented form Bashkim at Al [52]. This allows a near real time monitoring of hROS in brain through rat striatal microdialysis system thanks to the highly selective fluorescence of OH-TA. This approach provides several significant advantages over HPLC in terms of its simplicity, sensitivity, reproducibility and near real time conditions. In comparison to the HPLC analysis of collected perfusates, the real time method has the advantage of resolving also small and short lasting OH-TA peaks. This may be useful for possible future human application. Furthermore, while the use of the HPLC is limited in terms of time resolution (sampling time cannot be reduced below 10 min), the on line method provides real-time information for monitoring of hROS under physiological and pathological conditions and should be extremely helpful to further investigate and understand the role of hROS in the pathogenesis of neurological disorders and to develop medical strategies to reduce the damaging potential of hROS.

The detection limits of the on line and HPLC measurements presented form Bashkim at Al [52], were 1,18 nM and 0,98 nM, respectively, indicating the same sensitivity for the two methods. Furthermore, it can be concluded from the data presented below that the online method allows a more accurate quantitation of hROS. As HPLC data represent the mean over a 20 min collection period, especially short lasting hROS releases can be significantly higher than quantified by HPLC. This is shown in *Scheme 1.4 and 1.5* were two types of on line recordings in comparison to HPLC quantitation are schematically presented: In the first case a larger hROS releases is schematically presented (a longer-duration peak of 40 min see *Scheme 1.4*) this on line peak is measured on HPLC in two fractions, therefore the HPLC peak of each fraction is only 1/2 of the total area of on line peak. This behavior is normally due to the dilution, where 10 min of on line peak for each fraction is represented as basal peak. Whereas in the second case a small short lasting hROS releases schematically is presented (a short-duration peak of 5 min see *Scheme 1.5*) where due to the dilution the HPLC peak is only 1/3 of the total area of on line peak. Therefore, in order to address this issue it was of primary importance to know exactly the time at which the hROS peak can be integrated, therefore in order to find out this it was necessary to have a strict control over the on line protocols.



Scheme 1.4. Comparison between on line recordings and HPLC quantitation. The red lines represents the on line releases, whereas the blue square represents the HPLC peaks measured in two fractions



Scheme.1.5. Comparison between on line recordings and HPLC quantitation. The red lines represents the short lasting hROS releases whereas the blue square represents the same on line peak measured with HPLC

Conclusions

Unlike hydrogen peroxide and superoxide, which are destroyed by catalase, peroxidases, and the superoxide dismutases, there are no known enzymes that catalyze the removal of hROS. Thus, their formation and removal are rapid but unregulated. Much of the work relating oxidative stress to disease has been based on determining the effects in terms of DNA, lipid and protein damage [53, 54, 55]. However, since these measure the end-products of ROS damage, they will be affected by factors such as the efficiency of local antioxidant defenses and repair mechanisms. These can show considerable inter-individual variability, depending on diet, disease status, age, and genetic factors [56, 57]. In view of the wide-ranging toxicity of hROS, it is difficult to envisage specific regulatory functions, although singlet oxygen has been reported to regulate plastid differentiation in plant seedlings [58]. Further advances in our understanding necessitate the application of sensitive and reliable methods for hROS detection in vivo, such as those discussed in this review. Their use in microdialysis experiments to detect hROS in vivo, in freely moving animals, has considerable potential for research and medicine [see 59, 60]. However, caution and rigor are essential for the correct interpretation of the data obtained. Thus, high basal hROS levels have been reported in some [see, 61, 62], but not all [59] microdialysis studies in the brain and also in studies with isolated cell preparations [63].

It has been reported that hROS formation might be catalyzed by iron leaking from the stainless-steel probes that are used in many microdialysis studies [64]. It has also been suggested that hROS formation might result from cell damage caused by the probe insertion [65]. Clearly this effect would be an interesting field of study, but the use of adequate recovery times after probe insertion should minimize this response. The alternative possibility that the formation and release of hROS under basal conditions is a normal physiological process warrants further investigation. There are still numerous unsolved questions related to hROS formation and activities in vivo as well as in vitro. In addition to the further development of analytical techniques, a multi-causal and interdisciplinary approach will be necessary for a resolution of many of these questions.

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