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Oxidation of Vitamin E, Vitamin C, and Thiols in Rat Brain Synaptosomes by Peroxynitrite

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ABSTRACT. Peroxynitrite is formed by the reaction of superoxide with nitric oxide, an important neurotransmitter. Incubation of rat brain synaptosomes with peroxynitrite resulted in the consumption of antioxidant substances such as α-tocopherol, ascorbate, and thiols. Membrane cholesterol was not oxidized under the same conditions. α-Tocopherol and ascorbate in synaptosomes were oxidized very rapidly by peroxynitrite. In contrast, previous reports in the literature have shown that peroxynitrite treatment did not oxidize tocopherol in human plasma. Peroxynitrite in sufficient concentrations oxidized all of the tocopherol and ascorbate in synaptosomes. Thus, the oxidant is able to diffuse to the different membranes in synaptosomes and oxidize tocopherol in all of them. α-Tocopherol is converted quantitatively to tocopherolquinone during the oxidation. Significant amounts of thiols (at least 30% of the total thiols) do not seem to be accessible to oxidation by peroxynitrite. However, the concentration of thiols is much higher than those of tocopherol and ascorbate. Addition of the hydroxyl radical quenchers benzoate or mannitol or the enzymes superoxide dismutase or catalase (alone or together) did not affect the oxidation of tocopherol and ascorbate by peroxynitrite, whereas cysteine and glutathione blocked the oxidation. Therefore, reactive oxygen species may not be directly involved as intermediates in oxidations induced by peroxynitrite. The latter is a potent oxidizing agent that can oxidize substances such as tocopherols, ascorbate, and thiols in the immediate vicinity of its formation. The antioxidant nutrients ascorbate and tocopherol could play important roles in protecting brain from oxidative damage induced by peroxynitrite. BIOCHEM PHARMACOL 52;4:579–586, 1996.

KEY WORDS. vitamin E; vitamin C; thiol; peroxynitrite; synaptosomes; antioxidants; oxidation

Most aerobic cells are capable of forming superoxide as a secondary product during respiration or from specific enzymatic reactions. Similarly, a variety of cells including neurons, endothelial cells, neutrophils, and macrophages produce nitric oxide which has been shown to be a neurotransmitter in the nervous system [1]. It is well known that nitric oxide and superoxide can combine to form peroxynitrite, an unstable product with a half-life of 1 or 2 sec [2]. The rate of reaction of superoxide with nitric oxide is quite fast with a rate constant of 6.7×10^9 mol^{-1} sec^{-1} [3]. It was postulated originally that this reaction could be responsible for the quenching of superoxide and thus eliminate the toxicity of superoxide and related oxygen free radicals. However, data indicate that peroxynitrite itself is a very potent oxidizing agent, and therefore the reaction between superoxide and nitric oxide produces a new and powerful oxidizing substance [3].

The cell contains a number of antioxidants that protect it against oxidative injury. These can be categorized into two general groups: (a) macromolecular proteins (enzymes) such as superoxide dismutase, glutathione peroxidase, and catalyse, and (b) smaller molecular weight substances such as ascorbic acid, glutathione, and vitamin E. Our goal in this investigation was to determine the rate of loss of selected antioxidants when a subcellular fraction from brain (synaptosomes) was exposed to peroxynitrite. The primary focus is on the oxidation of vitamin E, but we have also examined oxidation of vitamin C and thiols as well. Synaptosomes are produced during homogenization of brain and represent the nerve endings that are pinched off during homogenization. This subcellular fraction has been extremely valuable for studying the mechanisms of neural transmission.

MATERIALS AND METHODS

Chemicals

The chemicals used were of reagent grade purity from standard sources. Solvents for chromatography were HPLC grade from Burdick & Jackson Laboratories, Inc., Muskegon, MI. Other special chemicals were purchased from the following sources: α-tocopherol and α-tocopherolquinone

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from Kodak Laboratory Chemicals, Rochester, NY; Ficoll (Type 400) from the Sigma Chemical Co., St. Louis, MO. Absolute ethanol was obtained from the Midwest Solvents Co., Pekin, IL, and was redistilled prior to use. Most of the reagent grade chemicals were from Sigma.

Experimental Procedures

All experiments were repeated and confirmed with brain samples from separate animals on different days. Data from typical experiments are reported.

Preparation of Peroxynitrite

Peroxynitrite was prepared by the method of Keith and Powell [4]. Briefly, a mixture of sodium nitrite and hydrogen peroxide was acidified with hydrochloric acid, and almost immediately sodium hydroxide was added to neutralize the acid and make the solution alkaline. The excess hydrogen peroxide was destroyed by passing the solution through manganese dioxide. The solution was then frozen. A darker yellow solution enriched in peroxynitrite separated out, was removed, and was used in all experiments. The peroxynitrite concentration was estimated using an extinction coefficient of 1670 mol^{-1} cm^{-1} at 302 nm [5]. The peroxynitrite solution prepared is usually very basic. When larger volumes of peroxynitrite were added, part of the excess base was neutralized on the day of the experiment. This peroxynitrite solution of lower basicity was checked for its absorption at the beginning and end of the study to ascertain that the peroxynitrite had not decomposed during the time required for incubations. The incubation tubes were also routinely checked to make sure that the final pH did not change after the addition of peroxynitrite.

Preparation of Subcellular Fractions from Rat Brain

Four-month-old, male Fisher 344 rats were used. The subcellular fractions were isolated from cerebral hemispheres by standard centrifugation methods [6]. The Ficoll solution used was purified by dialyzing a 40% (w/v) aqueous solution against water for 3 hr. The final Ficoll concentration was estimated by using a graph relating density and concentration. Brain tissue was homogenized in 10 vol. of ice-cold isolation medium containing 0.32 M sucrose, 10 mM HEPES, and 1 mM EDTA at pH 7.4, using a Dounce homogenizer. The homogenate was centrifuged at 1300 *g* for 3 min, and the supernatant was saved. The pellet was resuspended in 10 mL of the isolation medium, rehomogenized, and centrifuged at 1300 *g* for 3 min. The pooled supernatants were centrifuged at 17,000 *g* for 10 min to get the crude mitochondrial fraction. The resulting pellet was resuspended in 15 mL of isolation medium. Half of this suspension was layered over 11 mL of 7.5% (w/v) Ficoll medium that had been layered over 11 mL of 10% (w/v) Ficoll medium. The tubes were centrifuged in a Beckman SW 28 rotor at 99,000 *g* for 45 min. The fraction at the interface between the two Ficoll solutions was removed, diluted 1:5 with isolation medium, and centrifuged for 10 min at 17,000 *g* to isolate the synaptosomes. The purity of the synaptosomal fraction was tested by electron microscopy and also by estimating the activities of the marker enzyme of Na^+-K^+-ATPase [7]. The activity of the ATPase was usually enriched 3-fold in synaptosomes compared with the crude homogenate.

Protocol for Incubations

Synaptosomes were incubated at 37° in a medium (pH 7.4) simulating extracellular fluids or plasma and had the following composition: 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium phosphate, and 10 mM glucose. HEPES (4 mM) was also added for additional buffering capacity. Test tubes containing buffer and the synaptosomal fraction were equilibrated at 37° for 10 min, and the peroxynitrite was added while mixing. After an additional 10 min of incubation at 37°, the tubes were placed on ice. The mixture was then centrifuged for 30 min at 17,000 rpm in a Sorvall SS 34 rotor (35,000 *g*) to sediment the synaptosomes. The sedimented fractions were then analyzed for various components.

Since peroxynitrite decomposes spontaneously in buffer within a few seconds, it was possible to run peroxynitrite-depleted controls. These control tubes were incubated with peroxynitrite for 10 min to decompose all of the peroxynitrite, and then the synaptosomal samples were added. The samples are referred to as peroxynitrite-depleted controls.

Determination of Ascorbate

All samples for ascorbate analyses were mixed with metaphosphoric acid (5% final concentration) containing 0.1% cysteine and 1 mM EDTA and centrifuged at 1500 *g* for 10 min. The supernatant containing the ascorbate was removed and kept at −70° until analysis. Ascorbate was determined by liquid chromatography with electrochemical detection by the method of Margolis [8]. The chromatographic conditions were as follows: Aminex HPX 87H cation exchange column, 300 × 7.8 mm (Bio-Rad Laboratories, Richmond, CA); eluent, 5% acetonitrile in 0.1 M formic acid; flow rate, 0.5 mL/min; detector, Coulochem model 5100A (ESA Inc., Bedford, MD) with the D2 electrode of the 5011 analytical cell set at 0.6 V.

Determination of Tocopherol, Tocopherolquinone, and Cholesterol

The method for determination of tocopherols and quinone by liquid chromatography has been published [9, 10].

Briefly, 2 mL ethanol containing 0.025% (w/v) BHT* and 0.1 mL of 30% (w/v) ascorbic acid were pipetted into tubes containing samples for tocopherol analyses. The mixture was saponified at 60° for 30 min after the addition of 1 mL of 10% potassium hydroxide solution. Tubes were cooled, and 2 mL of water was added followed by 2 mL of hexane containing 0.025% (w/v) BHT. Tocopherols and quinone were extracted into the hexane phase by vortexing for 1 min. The hexane phase was separated out and evaporated down under a stream of nitrogen. The residue was redissolved in mobile phase and analyzed by reverse phase liquid chromatography using the following conditions: column = ultrasphere ODS, 5 μm, 4.6 × 150 mm (Beckman Instruments); mobile phase = methanol:water, (94.5:5.5) with 7.5 mM sodium dihydrogen phosphate (final concentration); flow rate = 2.7 mL/min. The tocopherols and tocopherol-quinone were detected electrochemically: Coulochem 5100 A detector, 5011 analytical cell with detector 1 at −0.25 V and detector 2 at +0.55 V, and 5021 conditioning cell at −0.75 V.

One portion of the hexane extract was used for cholesterol determinations. After evaporation of the solvent, trimethylsilyl ether derivatives of cholesterol were prepared using bis(trimethylsilyl) trifluoroacetamide and analyzed by GC equipped with a flame ionization detector and a capillary column [DB1 column, 30 m × 0.324 mm, film thickness 0.1 μm; temperature programming conditions—oven temperature initial 50°, initial time 3 min, program rate 30°/min, final value 230°, second program rate 4°/min, final temperature 310° and keep at 310° for 3 min]. Under these conditions, the retention times of the internal standard cholestane and cholesterol were 17.43 and 21.40 min, respectively.

Biochemical Assays

The concentration of total protein was determined by the Lowry technique as modified by Markwell *et al.* [11]. The concentrations of total sulfhydryl groups in the samples were determined by the method of Habeeb [12]. The samples were dissolved in 0.08 M sodium phosphate buffer (pH 8.0) containing EDTA (0.5 mg/mL) and sodium dodecyl sulfate (2%) and then treated with 5,5′-dithiobis-(2-nitrobenzoic acid). The absorbance of the solution was measured at 410 nm. Sulfhydryl concentrations were calculated using glutathione as the standard.

Hydrogen peroxide in the peroxynitrite was determined by the modified iodometric procedure of Darrow and Organisciak [13]. This technique is reliable for measurement of peroxide in purified solutions of hydroperoxides or peroxides, but not in biological extracts since the latter contains antioxidants that interfere with the assay. The peroxynitrite solution was acidified, the active compound was allowed to decompose, and then hydrogen peroxide in the solution was assayed. The hydrogen peroxide concentrations in the peroxynitrite solutions were always less than 1% of the peroxynitrite.

RESULTS

The stability of peroxynitrite varies greatly with pH. In one experiment, we incubated synaptosomes with peroxynitrite in media at varying levels of pH. The oxidation of vitamin E was followed. HEPES buffer was used between pH 7 and 8.2, and PIPES was used at the lower pH values. Figure 1 shows the dependence of tocopherol oxidation upon pH. The pH optimum for tocopherol oxidation was fairly broad, ranging from pH 7 to 7.8. Similarly, it was observed that the pH optimum for ascorbate oxidation was 7 to 8. All further experiments were conducted at physiological pH of 7.4.

The rate of oxidation of α-tocopherol by peroxynitrite was examined in the next experiment. The results in Fig. 2A show that nearly all of the oxidation had taken place by the earliest sampling time of 30 sec. In a separate experiment, it was found that all of the ascorbate oxidation was also completed within the same time (Fig. 2B). An incubation time of 10 min was experimentally convenient for the handling of multiple samples, and this time was chosen for all the remaining experiments.

The interpretation of data on synaptosomal ascorbate concentrations after peroxynitrite treatment is somewhat

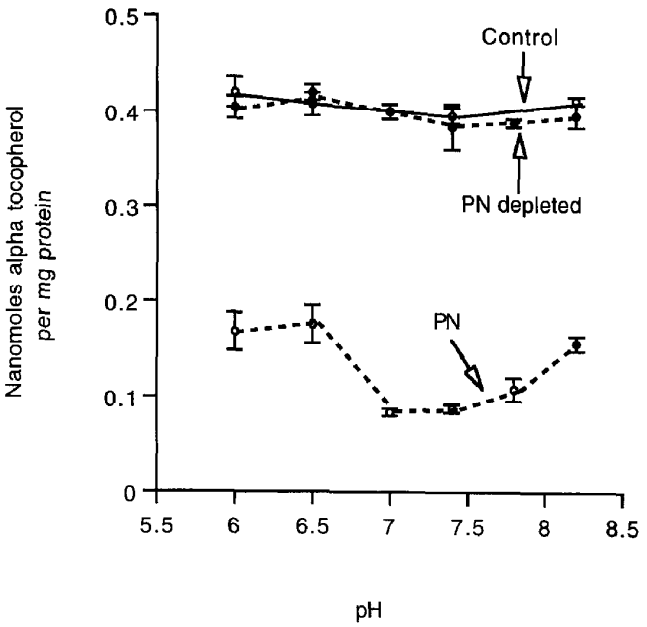

FIG. 1. Influence of the pH of incubation upon oxidation of α-tocopherol in rat brain synaptosomes. Synaptosomes from 4-month-old, male, Fischer 344 rat brain cerebral hemispheres were isolated by standard centrifugation techniques and incubated with peroxynitrite (PN, 2 mM) at 37° for 10 min. The protein content was 250 μg/mL, and the incubations were done in Krebs buffer with additional PIPES or HEPES. The experimental values are means ± SD (N = 3).

* *Abbreviations*: BHT, butylated hydroxytoluene; and PIPES, 1,4-piperazinediethanesulfonic acid.

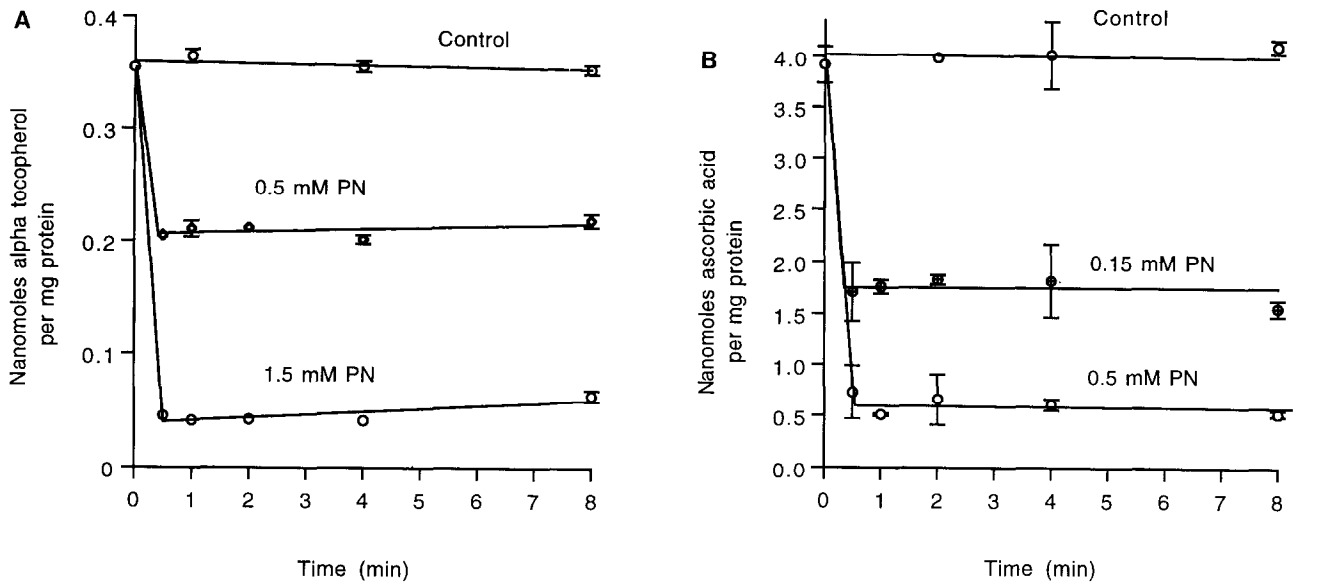

FIG. 2. Effect of time of incubation upon the oxidation of α-tocopherol and ascorbate in rat brain synaptosomes. Synaptosomes (250 μg/mL protein) from 4-month-old, male, Fischer 344 rat brain cerebral hemispheres were isolated by standard centrifugation techniques and incubated in Krebs buffer at 37° with peroxynitrite (PN) for different periods of time. (A) α-Tocopherol oxidation. (B) Ascorbic acid oxidation. The experimental values are means ± SD (N = 3).

complicated. Ascorbate appears in the medium when synaptosomes are incubated with buffer. (Interestingly, neither α-tocopherol nor thiols appear to be released into the medium.) The ascorbate released can be oxidized by either peroxynitrite or nitrite, which is present in the peroxynitrite-depleted control solutions. However, the ascorbate within the synaptosomes will be oxidized only during the incubations since the reaction mixture is centrifuged to recover the synaptosomes and the medium is separated and discarded. The ascorbate concentrations reported in this paper represent the total ascorbate that was found in the synaptosomes and the supernatant. Nonetheless, the differences in ascorbate concentrations between the synaptosomes treated with peroxynitrite or peroxynitrite-depleted solutions do reflect oxidation induced by peroxynitrite alone.

The influence of peroxynitrite concentration upon oxidation of synaptosomal α-tocopherol was determined in the next experiment. All incubations were done as usual in modified Krebs buffer at pH 7.4 and 37°. It can be seen from Fig. 3 that nearly all of the α-tocopherol was oxidized when synaptosomes were incubated with 5 mM peroxynitrite. The production of tocopherolquinone during the oxidation of synaptosomal α-tocopherol by peroxynitrite was also monitored. The results from a typical experiment (Fig. 4) show that almost all of the tocopherol oxidized is recovered as tocopherolquinone. In our experience, peroxynitrite was the only oxidant that produced quantitative yields of tocopherolquinone during *in vitro* oxidations. Less than 50% of tocopherol oxidized was converted to tocopherolquinone during oxidations induced by other oxidants such as iron plus ascorbate, iron plus fatty acid hydroperoxide, and synthetic free radical generators.

The oxidation of synaptosomal ascorbate is shown in Fig. 5. There were two differences between α-tocopherol and ascorbate oxidations induced by peroxynitrite: (a) all of the ascorbate was oxidized by a lower concentration of peroxynitrite than α-tocopherol oxidation, and (b) the peroxynitrite-depleted solutions were capable of oxidizing some of the ascorbate in synaptosomes. The concentrations of per-

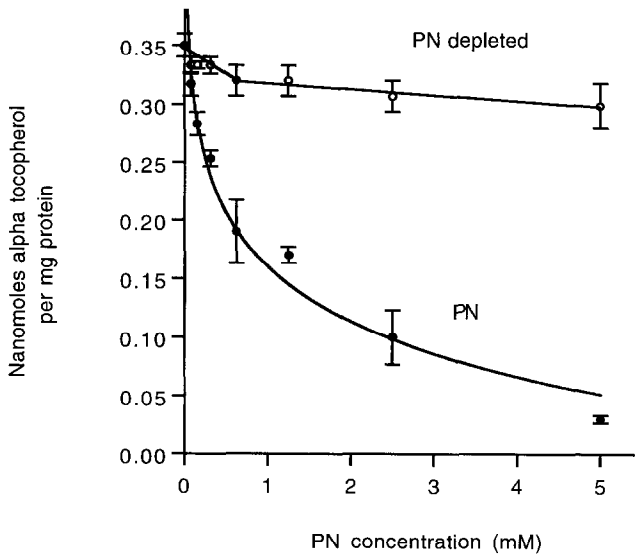

FIG. 3. Oxidation of α-tocopherol in rat brain synaptosomes by peroxynitrite (PN). Synaptosomes (250 μg/mL protein) from 4-month-old, male, Fischer 344 rat brain cerebral hemispheres were isolated by standard centrifugation techniques and incubated in Krebs buffer at 37° for 10 min with various concentrations of PN. The experimental values are means ± SD (N = 3).

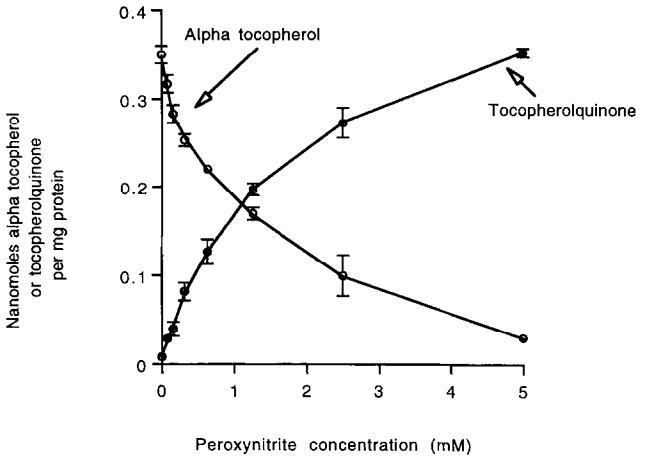

FIG. 4. Oxidation of α-tocopherol with simultaneous generation of tocopherolquinone during treatment of synaptosomes with peroxynitrite. Synaptosomes (250 μg/mL protein) from 4-month-old, male, Fischer 344 rat brain cerebral hemispheres were isolated by standard centrifugation techniques and incubated in Krebs buffer at 37° for 10 min with various concentrations of peroxynitrite. The experimental values are means ± SD (N = 3).

oxynitrite required to achieve 50% oxidations of ascorbate and α-tocopherol were 0.3 and 0.8 mM, respectively.

The oxidation of thiols by peroxynitrite was also examined in a similar experiment, and the results are shown in Fig. 6. The data indicate that when compared with α-tocopherol and ascorbate, total thiols were somewhat resistant to oxidation by peroxynitrite. In a separate experiment, synaptosomes were incubated with a high concentration of peroxynitrite (15 mM), and it was found that 70% of the total thiols were oxidized. Thus, a substantial portion of the total thiols remained unoxidized by peroxynitrite. Some oxidation of thiols took place even with peroxynitrite-depleted controls (Fig. 6). However, peroxynitrite caused additional oxidation of sulfhydryls. A comparison of thiol oxidation with data in Figs. 3 and 5 shows that whereas all of the tocopherol and ascorbate were oxidized by peroxynitrite, only about 70% of the sulfhydryls were available for oxidation at the high peroxynitrite concentration of 15 mM.

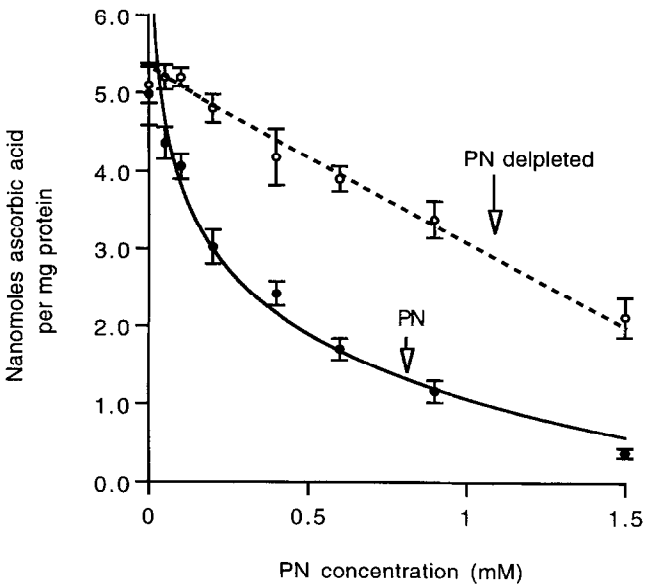

FIG. 5. Oxidation of ascorbic acid during treatment of rat brain synaptosomes with peroxynitrite (PN). Synaptosomes (250 μg/mL protein) from 4-month-old, male, Fischer 344 rat brain cerebral hemispheres were isolated by standard centrifugation techniques and incubated in Krebs buffer at 37° for 10 min with various concentrations of PN. The experimental values are means ± SD (N = 3).

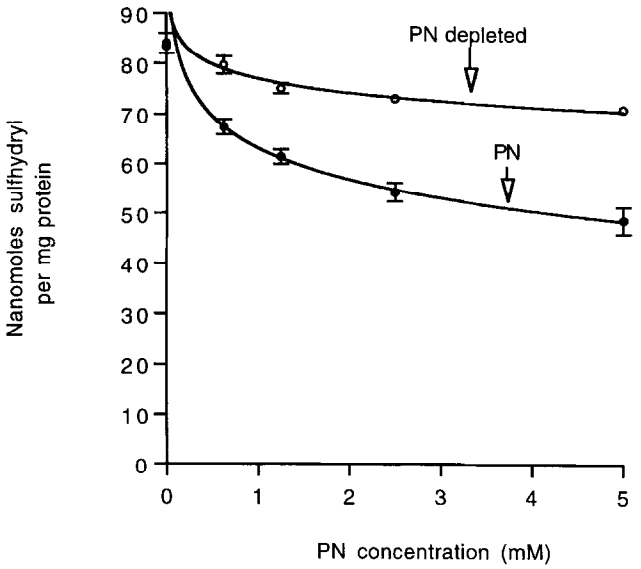

FIG. 6. Oxidation of total thiols during treatment of rat brain synaptosomes with peroxynitrite (PN). Synaptosomes (250 μg/mL protein) from 4-month-old, male, Fischer 344 rat brain cerebral hemispheres were isolated by standard centrifugation techniques and incubated in Krebs buffer at 37° for 10 min with various concentrations of PN. The experimental values are means ± SD (N = 3).

To understand the mechanism of oxidation of α-tocopherol and ascorbate by peroxynitrite, the effect of the addition of inhibitors of oxyradicals upon the oxidation was studied. The results are given in Fig. 7. Benzoate and mannitol, which are known to quench hydroxyl radicals, did not block the oxidation of tocopherol or ascorbate. Thus, hydroxyl radicals were not responsible for the oxidation of tocopherol or ascorbate. The addition of cysteine and glutathione blocked the oxidations of both tocopherol and ascorbate. This blocking of oxidation by cysteine and glutathione may be due to the direct reaction of the compounds with peroxynitrite.

The effect of the addition of the antioxidant enzymes superoxide dismutase and catalase to the incubation medium before the addition of peroxynitrite was tested in the next experiment. In these experiments, superoxide dismutase or catalase (200 U of each or a mixture of the two per

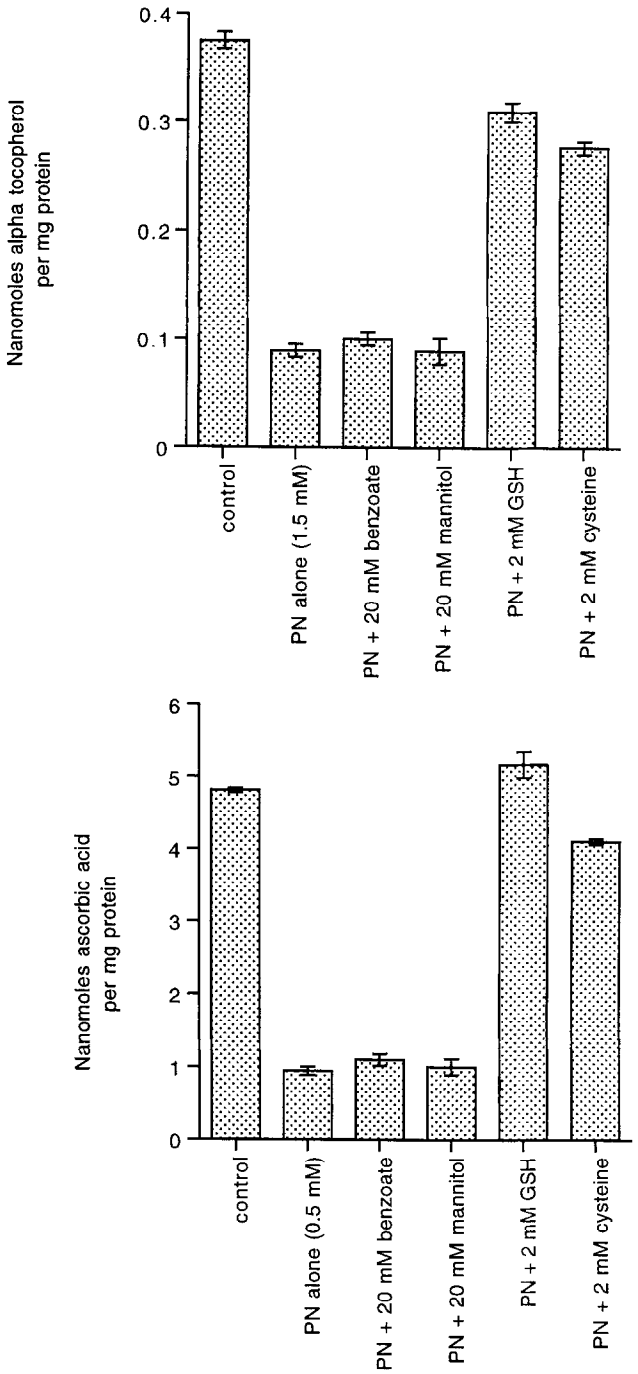

FIG. 7. Effect of addition of benzoate (20 mM), mannitol (20 mM), cysteine (2 mM), and glutathione (GSH, 2 mM) upon the oxidation of α-tocopherol and ascorbic acid in rat brain synaptosomes incubated with peroxynitrite (PN). The concentrations of PN used were 1.5 and 0.5 mM for α-tocopherol and ascorbate oxidations, respectively. Synaptosomes (250 μg/mL protein) from 4-month-old, male, Fischer 344 rat brain cerebral hemispheres were isolated by standard centrifugation techniques and incubated in Krebs buffer at 37° with PN for 10 min. The experimental values are means ± SD (N = 3).

mL of the incubation medium) was added. It was found that the addition of the enzymes alone to control synaptosomes had no effect upon the ascorbate or α-tocopherol concentrations. When peroxynitrite (0.5 mM) was added after the addition of the enzymes, the extent of oxidation of ascorbate and α-tocopherol was not altered by the presence of the enzymes. Therefore, it seems likely that hydrogen peroxide, superoxide, or hydroxyl radicals (data from the previous paragraph) are not directly involved in the oxidations induced by peroxynitrite.

Even though catalase did not affect the oxidations induced by peroxynitrite, the potential of hydrogen peroxide to induce oxidations needs to be considered. This is especially important since hydrogen peroxide was one of the reagents used in the preparation of peroxynitrite. Most, if not all, of the hydrogen peroxide was removed by treatment with manganese dioxide. Nonetheless, it is conceivable that a small amount of the peroxide may be remaining in the peroxynitrite preparation. Therefore, the potency of hydrogen peroxide in oxidizing α-tocopherol in synaptosomes was tested. When a synaptosomal preparation (375 μg protein) was treated with 1 mM hydrogen peroxide for 2 hr, 20% of the α-tocopherol was oxidized and only 38% of the tocopherol oxidized was converted to tocopherolquinone. Similar data were also obtained upon repeating this experiment with another batch of synaptosomes. Hence, the extent of oxidation of α-tocopherol by hydrogen peroxide as well as the yield of tocopherolquinone is smaller than that with peroxynitrite. The presence of unreacted hydrogen peroxide could not be responsible for the oxidations reported in this paper.

In all of the experiments described above, the oxidation of cholesterol in synaptosomes was determined by assaying for concentrations of total cholesterol. There were no changes in the cholesterol concentrations, suggesting that cholesterol was resistant to oxidation by peroxynitrite under these conditions.

DISCUSSION

It is noteworthy that peroxynitrite oxidized nearly all of the tocopherol present in the synaptosomal fraction (Fig. 4). Peroxynitrite or an oxidant derived from it was able to react with tocopherol present in the membranes of synaptosomal mitochondria as well as in the plasma membrane. This is in sharp contrast with our earlier observation that other oxidants such as synthetic free radical generators are not able to oxidize a pool of tocopherol (about 20% of the total tocopherol) [14]. We considered this as an inaccessible pool of tocopherol. Even though the half-life of peroxynitrite is only a few seconds, it is able to penetrate different types of cellular membranes and oxidize the tocopherol in them. The entry of peroxynitrite into some membranous biological structures may be facilitated by the anion transporter present in many membranes. In addition, even though protonated peroxynitrite is very unstable, it may be transported across biological membranes in this form as well. Whatever the mechanism of entry of peroxynitrite into cells may be, it is clear from our data that peroxynitrite is capable of oxidizing substances such as α-tocopherol at sites away from the site of formation or release.

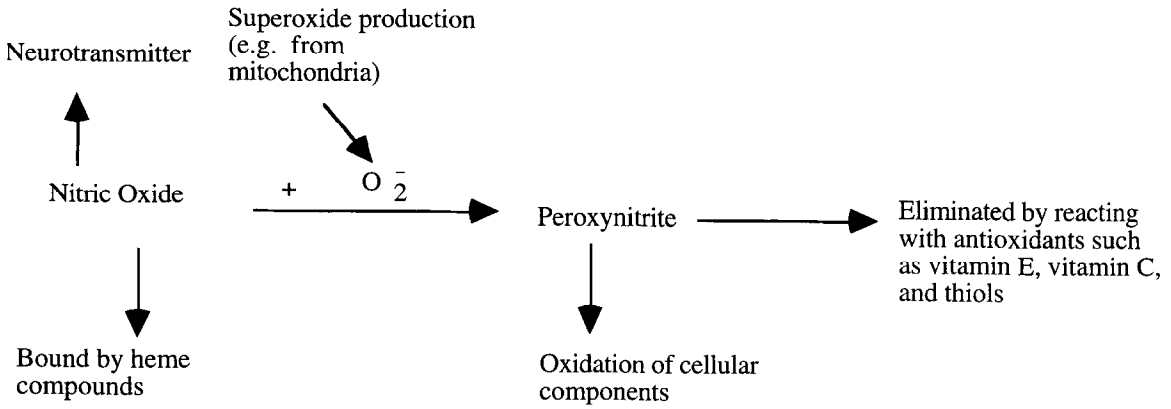

FIG. 8. Schematic representation of the formation and reactions of peroxynitrite.

The observation that tocopherolquinone is produced from α-tocopherol in near quantitative yield is worthy of further consideration. As mentioned earlier, this is the only instance where we have observed quantitative production of tocopherolquinone from tocopherol in biological samples. Graham *et al.* [15] have reported similar results during the oxidation of low density lipoproteins by peroxynitrite. These authors found experimental evidence (ESR) for the production of α-tocopheroxyl radicals during the oxidative reactions. A comparison of tocopherol oxidation by different oxidizing agents is important in this context. The extent of tocopherol oxidation, as well as the yield of the primary oxidation product (tocopherolquinone), was much lower during oxidations induced by hydrogen peroxide than those with peroxynitrite (see Results). With oxidants such as hydrogen peroxide and iron plus ascorbate, the tocopheroxyl radical is known to form adducts with other components reducing the yield of tocopherolquinone. In these cases, the yield of quinone is always less than 50% and the yield can vary from experiment to experiment. If tocopheroxyl radicals are formed during peroxynitrite-induced oxidations, it is difficult to explain why such side-reactions do not take place and reduce the yield of quinone. It is interesting to note that Hogg *et al.* [16] recently suggested that α-tocopheroxyl radicals are not involved in the oxidation of tocopherol by peroxynitrite. These authors studied the oxidation of tocopherol by peroxynitrite and proposed that these reactions involve α-tocopherone cation and not α-tocopheroxyl radical as an intermediate. Our data are consistent with this hypothesis.

The reactivity of peroxynitrite towards plasma antioxidants was investigated recently by Van Der Vliet *et al.* [17]. There are some similarities between the results obtained with plasma and those obtained with synaptosomes in the present study. Ascorbic acid is rapidly destroyed in both cases. However, thiol oxidation seemed to proceed to a much greater extent with plasma than with synaptosomes. In addition, Van Der Vliet *et al.* [17] found that α-tocopherol in plasma was not oxidized, whereas we observed that synaptosomal tocopherol was oxidized nearly completely by treatment with peroxynitrite. The differences between the two studies are interesting. It is possible that antioxidants are dispersed more homogeneously in blood plasma than in synaptosomes, which are structurally more complex than plasma. This may result in peroxynitrite reacting first with other antioxidants such as ascorbate and urate that are present in high concentrations in plasma before reacting with tocopherols.

Our data show that both vitamins C and E are capable of reacting with and neutralizing peroxynitrite. The presence of adequate levels of vitamins C and E would aid in destroying peroxynitrite at the site of its formation before it has a chance to attack critical biological molecules. Therefore, high cellular concentrations of these antioxidant vitamins could be of benefit in preventing oxidative damage induced by peroxynitrite.

The oxidation of thiols by peroxynitrite differed from the oxidations of ascorbate and tocopherol. Even when the concentration of peroxynitrite in the incubation medium was increased to 15 mM, a substantial portion (about 30%) of the total thiols remained unoxidized. However, the amount of thiol oxidation was quantitatively very significant since the steady-state levels of thiols in cells were much higher than those of vitamins C and E. Radi *et al.* [18] have studied the oxidation of cysteine and sulfhydryl groups in albumin by peroxynitrite. They have found that the rate constants for oxidation are three orders of magnitude larger than those with hydrogen peroxide. The authors also observed that peroxynitrite anion, not the protonated species, was the active agent reacting with thiols. The synaptosomal fraction contains smaller molecular weight thiols such as glutathione as well as protein thiols. In this study, we followed the oxidation of total thiols and did not differentiate between the various thiol species.

The high potency and the very rapid oxidation of vitamins C and E by peroxynitrite are similar to oxidations induced by hydroxyl radicals. Some investigators have suggested that peroxynitrite releases hydroxyl radicals and that the latter species causes the oxidations. However, others have disputed this theory and have preferred to call the active species "hydroxyl radical like." Our data on the effect of various inhibitors upon the oxidations induced by peroxynitrite (Fig. 7) show that reactive oxygen species are not responsible for the oxidative reactions. We have also

found that membrane cholesterol is not oxidized when synaptosomes are incubated with peroxynitrite concentrations of up to 15 mM. Furthermore, oxysterols were not formed during this treatment with 15 mM peroxynitrite (data not shown). This is in contrast to oxidation of cholesterol by a mixture of iron plus ascorbate where oxysterols are produced (Vatassery GT, unpublished observations). Thus, it seems unlikely that hydroxyl radicals are directly involved in the oxidations. The hypothesis of Koppenol *et al.* [3] that the *cis* form of peroxynitrite is the reactive intermediate is quite attractive since this does not call for the formation of hydroxyl radical as an intermediate.

Some of the reactive species formed during the heterolysis of peroxynitrite are $\cdot OH$, $\cdot NO_2$ and NO_2^+. Even though the hydroxyl radical formation may not be involved, the latter two species could be intermediates in the oxidations induced by peroxynitrite. The reactivity of peroxynitrite towards the various antioxidants is possibly limited only by the physical proximity and availability of the antioxidant since all antioxidants are oxidized by peroxynitrite.

Cells produce superoxide as a byproduct of respiration and from enzymatic reactions. It has been well established that neurons are also capable of producing nitric oxide. Astrocytes and microglia in culture have also been shown to produce nitric oxide [19]. Nitric oxide is a relatively long-lasting free radical and would be able to traverse considerable distances intra- or extracellularly. At the sites of production of superoxide (e.g. mitochondrial or endoplasmic reticulum), nitric oxide will combine with superoxide and produce the potent oxidant peroxynitrite. Our data show that peroxynitrite is capable of diffusing to distant parts of synaptosomes and reacting with different antioxidants. It is also possible that peroxynitrite may be able to provide a biological signal through oxidation of components such as sulfhydryl groups in critically important proteins. In other words, nitric oxide either can be eliminated by combining with heme compounds or reacts with superoxide producing peroxynitrite and triggering an oxidative sequence. The endogenous nutritional antioxidants vitamins C and E modulate the activity of peroxynitrite by reacting with and neutralizing it. It is important to recall that ascorbate and α-tocopherol react rapidly with peroxynitrite (see Results and Fig. 2) so that the nutrients can react with peroxynitrite almost as soon as the latter is liberated. A schematic representation of the reactions of peroxynitrite and their potential modulation by vitamins C and E is shown in Fig. 8. Clearly, the antioxidant nutrients vitamin E and vitamin C will be expected to play important roles in regulating the biological effects of peroxynitrite.

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