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5-Aminosalicylic Acid Protection against Oxidative Damage to Synaptosomal Membranes by Alkoxyl Radicals In Vitro

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The antioxidant properties of 5-aminosalicylic acid in vitro were evaluated in a synaptosomal membrane system prepared from gerbil cortical synaptosomes using EPR spin labeling and spectroscopic techniques. MAL-6 (2,2,6,6-tetramethyl-4-maleimidopiperidin-1-oxyl) and 5-NS (5-nitroxide stearate) spin labels were used to assess changes in protein oxidation and membrane lipid fluidity, respectively. Synaptosomal membranes were subjected to oxidative stress by incubation with 1 mM azo-bis(isobutyronitrile) (AIBN) or 1 mM 2,2′-azobis(amidino propane) dihydrochloride (AAPH) at 37°C for 30 minutes. The EPR analyses of the samples showed significant oxidation of synaptosomal proteins and a decrease in membrane fluidity. 5-Aminosalicylic acid also was evaluated by means of FRAP (the ferric reducing ability of plasma) test as a potential antioxidant. 5-Aminosalicylic acid also showed protection against the oxidation in gerbil cortical synaptosomes system caused by AIBN and AAPH. These results are consistent with the notion of antioxidant protection against free radical induced oxidative stress in synaptosomal membrane system by this agent.

KEY WORDS: Reactive oxygen species; EPR; 5-aminosalicylic acid; (5-ASA), 2,2′-azobis(amino propane) dihydrochloride; (AAPH); azo-bis(isobutyronitrile); (AIBN); synaptosomal membranes; antioxidants.

INTRODUCTION

Oxidative stress, a disturbance of the balance between pro- and anti-oxidants, is believed to be a chief force underlying some neurodegenerative disorders, and it has been implicated in age-related diseases such as Alzheimer's disease (AD) (1,2) and amyotrophic lateral sclerosis (ALS) (3). Furthermore, oxidative stress plays a crucial role in the process of aging (4). Reactive oxygen species (ROS), short lived, highly reactive chemical moieties, are the main cause of oxidative stress conditions. ROS such as peroxynitrite, and hydroxyl, peroxyl, and superoxide radicals react with lipids, protein, and DNA causing oxidative modifications. It has been shown by Stadtman's laboratory that free radicals can oxidize, and thus alter, the functionality of the proteins via addition of carbonyl groups to the side chain of the amino-acid molecules (4). Reactive oxygen species can also change functionality of membranes by reacting with polyunsaturated fatty acids and causing the breakdown of the fatty acid chain causing formation of 4-hydroxy-2-trans-nonenal

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Abbreviations: 5-ASA, 5-amino salicylic acid; FRAP (the ferric reducing ability of plasma); AAPH, 2,2′-azobis(amidino propane) dihydrochloride; AIBN, azo-bis(isobutytonitrile); EPR, electron paramagnetic resonance; MAL-6, 2,2,6,6-tetramethyl-4-maleimidopiperidin-1-oxyl; 5-NS, 5-nitroxide stearate.

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(4-HNE), malondialdehyde, acrolein and other neurotoxic species that further contribute to the oxidative stress conditions (5,6).

Brain cells are especially susceptible to oxidative stress due to the lack of cell division, the large presence of polyunsaturated fatty acids (PUFA), and the relatively high concentration of redox metal ions, especially iron and copper, that can catalyze free radical formation via Fenton chemistry. In addition, the high oxygen consumption and low level of antioxidants make brain vulnerable to oxidative stress. Our laboratory and others have demonstrated some unfavorable oxidative modifications caused by production of hydroxyl radicals or peroxynitrite in brain cells (7–13) manifested by a high extent of protein oxidation and inactivation of key enzymes prone to oxidation, such as glutamine synthetase (GS) (14) and creatine kinase (CK) (15,16). Also the regional correspondence between AD histopathology and biomarkers of protein oxidation (17) and increased protein oxidation in models of Huntington's disease have been demonstrated (18,19).

One of the many therapeutic strategies against the damage caused by ROS and free radicals is to administer antioxidants that can effectively scavenge the toxic molecules without causing damage to the cells of organisms. This approach can either render harmless the free radicals, and thus prevent the unfavorable oxidative conditions, or moderate the extent of damage caused by ROS. Previous studies have shown that commonly available antioxidants such as vitamin E (11,12,20–22), glutathione (23) or synthetic antioxidants such as pyrrolopyrimidines (24) can prevent or stop the oxidation of the brain samples and extend the life of rodents or hippocampal cell cultures exposed to oxidative stress (25).

5-Aminosalicylic acid (5-ASA) has been used as a therapeutic agent for treatment of inflammatory bowel disease where oxidative damage has been implicated (26). The mechanism of protection of 5-ASA against ROS induced membrane damage is still under evaluation. It is believed that the phenoxyl radical formed by reaction of 5-ASA with ROS is stabilized by the pi bond in the amine group. The ability of 5-ASA to protect phosphatidylcholine liposomes against peroxidation has been evaluated in conjunction with α-tocopherol by other researchers (27). 5-ASA blocks oxidative stress in cardiomyocytes treated with adriamycin (28); however, the action of 5-ASA against oxidation of brain cells has been little studied.

In this study we evaluate the antioxidant properties of 5-ASA in solution and in synaptosomal systems. We investigate the extent of protection on membrane-bound proteins and lipid bilayer that are oxidized in vitro by peroxyradicals generated by 2′2′-azobis(2-amidinopropane hydrochloride) (AAPH) and azo-bis(isobutyronitrile) (AIBN) breakdown (29,30). Also, we report the ability of 5-ASA to prevent changes in the synaptosomal membrane lipid bilayer manifested by protection from lipid peroxidation and changes in membrane fluidity. Electron paramagnetic resonance (EPR) spectroscopy combined with protein or lipid specific labeling techniques were the primary methods employed.

EXPERIMENTAL PROCEDURE

All chemicals were purchased from Sigma-Aldrich unless stated otherwise. AAPH was obtained from Wako Chemicals.

FRAP (the Ferric Reducing Ability of Plasma) Test. The detailed description of the assay is described elsewhere (31). Briefly, 300 mM acetate buffer, pH = 3.6 was prepared; 10 mM of TPTZ (2,3,6-tripyridyl-s-triazine) was dissolved in 40 mM HCl. 20 mM $FeCl_3$ was prepared in H_2O, and 1 mM $FeSO_4$ was prepared in acetate buffer as the reference standard. 2mM of 5-ASA stock solution was prepared in water. The FRAP reagent was prepared fresh by adding 10 volumes of acetate buffer to 1 volume of TPTZ and 1 volume of Fe(III) solution. 150 μl of FRAP and 5 μl of 5-ASA ranging from 20 to 800 μM were added to the plate reader. The plate was heated at 37°C for 4 minutes. Then the absorbance at 593 nm was measured. Fe(II) was used as the reference with the concentrations 20, 100, 200, 500, 750, and 1000 μM. The value of the slopes for 5-ASA and Fe(II) curves were obtained and the 'activity' of 5-ASA was deduced by dividing the slope of the 5-ASA by that of Fe(II).

All animals were housed and treated according to protocols approved by the University of Kentucky Animal Care and Use Committee. Male Mongolian Gerbils (aged between 2 and 3 months) were kept under 12 hours light-dark cycle and were provided with optimal housing conditions and diet. The animals were sacrificed by decapitation, and the brain was isolated immediately. The cortex was placed in 0.32 M sucrose isolation buffer containing 4 μg/ml leupeptin, 4 μg/ml pepstatin, 5 μg/ml aprotinin, 20 μg/ml trypsin inhibitor, 2 mM ethyleneglycol bistetraacetic acid (EGTA), 2 mM ethylenediamine tetraacetic acid (EDTA), and 20 mM 4-(2-hydroxyethyl)-1-piperazineethene sulfonic acid (HEPES), pH = 7.4). Samples were then homogenized by passing in the tube a motor driven pestle 10 times. The homogenate tissue was then centrifuged at 1,500 *g* for 10 minutes. The supernatant was collected and spun at 20,000 *g* for 10 minutes. The pellet was removed and placed on the top of discontinuous sucrose gradient (0.85 M, pH = 8; 1 M, pH = 8; and 1.18 M pH = 8.5 sucrose solutions each containing 10 mM HEPES, 2mM EDTA, and 2mM EGTA) and spun in a Beckman L7-55 ultracentrifuge at 82,500 *g* for 2 hours at 4°C. The synaptosomal layer was collected at the 1 / 1.18 molar sucrose interface, and subsequently lysed and washed twice with a lysing buffer (2 mM EDTA, 2 mM EGTA, 10 mM HEPES, pH 7.4) for 10 minutes at 32,000 *g*, yielding synaptosomal membranes. Protein concentration was determined by the BCA or Lowry method and adjusted to a concentration of 2 mg/ml.

Synaptosomal membrane samples (2 mg/ml pellet) were preincubated with 5-ASA for 10 min at room temp and were treated with 1 mM AIBN, or 1 mM AAPH followed by incubation at 37°C for

30 minutes to initiate free radical production. Samples were then incubated at room temp for an additional 30 minutes, and then they were washed by spinning at 14,000 *g* for 3 minutes in an Eppendorf centrifuge 3 times. Samples were then labeled with a paramagnetic, protein-specific spin label [2,2,6,6-tetramethyl-4-maleimidopiperidin-1-oxyl (MAL-6)] solution (50 μg/ml) and allowed to react for at least 12 hours at 4°C. Samples then were pelleted in a refrigerated Eppendorf table-top centrifuge. The supernatant was removed and the pellet was resuspended in 1 ml of fresh lysing buffer. This washing cycle was repeated six times to remove the excess spin label. No EPR signal was observed in the supernatant of the last wash, indicating no soluble proteins were spin labeled in these procedures. This procedure has been extensively characterized previously (8,32).

For 5-nitroxide stearate (5-NS) studies, the pellets (2 mg/ml dissolved in the Locke's Buffer), that were treated similarly with 5-ASA and AAPH or AIBN, were placed in glass tubes containing a chloroform-free 5-NS film (6 μg of spin label/ml of sample) and incubated at room temp for 30 minutes.

EPR spectra were acquired on Bruker EMX spectrometer using the following parameters: microwave frequency, 9.77 GHz; microwave power, 20 mW; receiver gain, 1×10^5, modulation amplitude, 0.3 G, and time constant, 1.28 ms.

Statistics. ANOVA was employed to determine the statistical significance of the results. *P* values less than 0.05 were considered significant.

RESULTS

The FRAP test is a useful tool for testing the potential antioxidant power of certain chemicals, and thus indicating the possible suitability of these compounds for further tests in in vivo and in vitro experiments. 5-ASA has some non-specific antioxidant activity as shown by the FRAP test (Fig. 1). The activity of the 5-ASA, as deduced from the slope of the curve, is 2.21 relative to the Fe(II) standard alone, implying that 5-aminosalycilic acid has a sufficient redox potential to drive the Fe(III)-TPTZ + e / Fe(II)-TPTZ reaction. Based on this test, it is clear that 5-ASA can be considered as a choice for further experiments involving brain samples.

The oxidative modifications of synaptosomal membrane proteins by AIBN and AAPH were investigated by EPR spin labeling studies with MAL-6 (Fig. 2 and 3). MAL-6 is a sensitive probe for measuring the extent of oxidation in synaptosomal membrane bound proteins, and the relevant EPR parameter, the W/S ratio, has been described extensively previously (6–8, 17–19,23,24). Lower values of the W/S ratio, reflecting slower motion of the spin label from control, are always observed in oxidative stress conditions (6–8, 17–19,23,24). Both AIBN and AAPH (1 mM) apparently lead to protein oxidation in synaptosomal membranes as assessed by the W/S ratios of MAL-6. The decrease in the W/S ratio with respect to control samples was observed to be 8.2% ($p < 0.001$) and 9.1% ($p < 0.006$) for AIBN and AAPH, respectively, after 30 minutes of incubation at 37°C (to initiate the breakdown of free radical precursors and produce free radicals). Pretreatment of synaptosomes with 20 μM of 5-ASA, however, brings the W/S ratio to the control

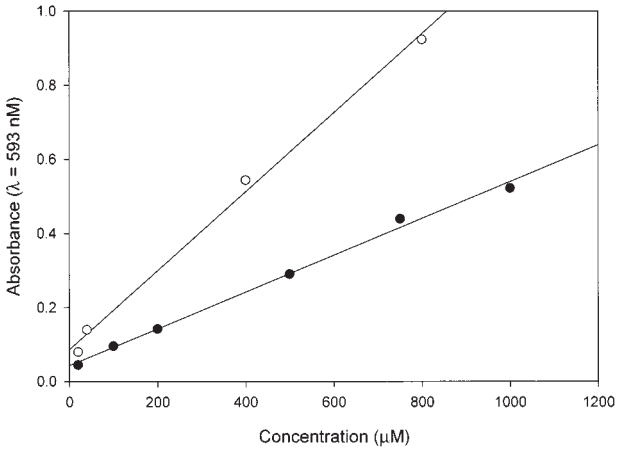

Fig. 1. Results for the FRAP assay for 5-ASA in solution. The response curve for 5-ASA (open circle) ($R^2 = 0.9954$) and for standard Fe(II) (filled circle) ($R^2 = 0.9949$). The activity of the antioxidant was determined from the slope ratios of 5-ASA and Fe(II) to be 2.21, indicating the antioxidant capacity of the compound. Each point represents the average of 4 measurements.

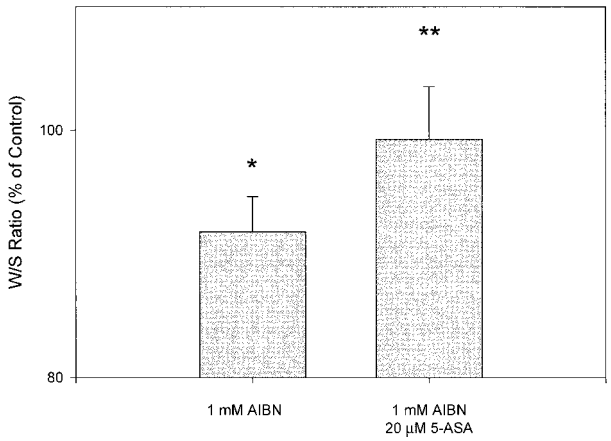

Fig. 2. W/S ratios of MAL-6 spin labeled synaptosomal membrane samples treated with 1 mM AIBN with or without 20 μM 5-ASA. 2 mg/ml samples were dissolved in the lysing buffer; 10 minutes of preincubation with 5-ASA was followed by 30 minutes incubation with AIBN at 37°C. Samples were then labeled with MAL-6 (50 μg/ml) and allowed to react for 12 h. Excess spin label was washed off by repeated centrifugations and resuspensions. Figures are normalized to the control values set as 100%. (*) $p < 0.001$ (for 1mM AIBN with respect to control samples), (**) $p < 0.02$ (AIBN vs. AIBN and 5-ASA treatment) n = 5–6.

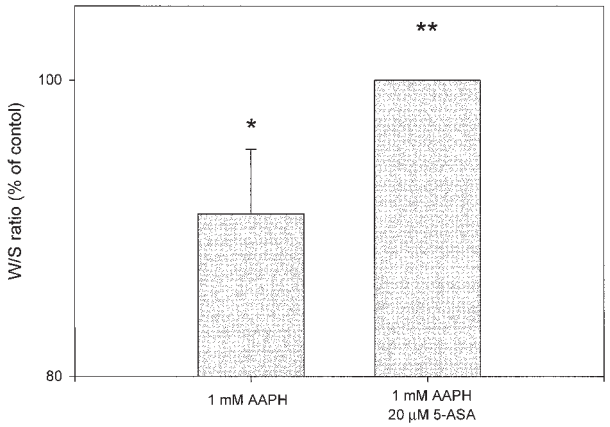

Fig. 3. W/S ratios of synaptosomal membrane samples treated with 1 mM AAPH with or without 20 μM 5-ASA. 2 mg/ml samples disolved in the lysing buffer; 10 minutes of preincubation with 5-ASA was followed by 30 minutes incubation with AIBN at 37°C. Samples were then labeled with MAL-6 (50μg/ml) and allowed to react for 12 h. Excess spin label was washed off. Figures are normalized to the control values set as 100%. (*) $p < 0.006$ (for 1 mM AAPH with respect to control samples), (**) $p < 0.008$ (1 mM AAPH vs. 1mM AAPH and 5-ASA treatment). n = 5–6.

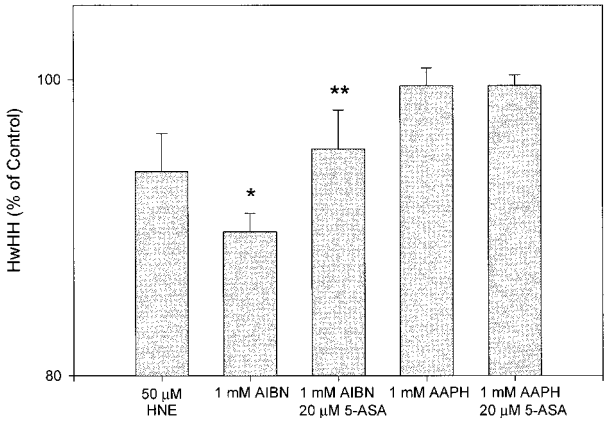

Fig. 4. Changes in HWHH of 5-NS labeled synaptosomal membranes. 2 mg/ml synaptosomes dissolved in Locke's buffer were preincubated with 20 μM 5-ASA for 10 minutes. Samples were then treated with 1 mM AAPH or 1mM AIBN. After 30 minutes of incubation samples were washed off, and dissolved in 400 μl of the Lock's buffer, and transferred to test tubes containing a thin film of 5-NS (6 μg of spin label/ml of sample) and incubated for 30 min. Values are shown as percentage of control. $p < 0.0001$ for AIBN (*) with respect to control samples; $p < 0.02$ for AIBN and AIBN + 5-ASA with respect to each other (**), n = 5–6.

level for both free radical generators, implying free radical protection by 5-aminosalicylic acid against protein oxidation (99.3% and 100% values of appropriate controls for AIBN and AAPH, respectively, $p < 0.008$ for both treatments, see Fig. 2 and 3 legends). The ratio of samples treated with 5-ASA alone with DMSO solvent were the same as controls. This result indicates that peroxyl radicals generated by AIBN and AAPH cause free-radical chain propagation reactions targeting the proteins and cause protein oxidation, and possible protein crosslinking, which could account for the decreased motion of the spin label observed.

In addition, alterations in the membrane lipid order and motion (fluidity) were assessed using the 5-NS spin label (Fig. 4). The relevant parameter is the half-width-half-height (HWHH), discussed previously in publications from our laboratory (24,33). Lower values of HWHH are associated with increased lipid order and decreased lipid motion, i.e., decreased fluidity. Samples treated with 1 mM AIBN showed a significant decrease in membrane bilayer fluidity caused by lipid peroxidation (89.7% of control values, $p < 0.001$). However, treatment with 1 mM AAPH produced no changes in membrane fluidity (99.6% of control values). The presence of 20 μM of 5-ASA partially protects the system from changes in fluidity caused by AIBN (95.3% of control values, $p < 0.02$). The vehicle solvent (DMSO) and 5-ASA alone do not cause any differences in membrane fluidity (data not shown).

DISCUSSION

Thermal decomposition of AAPH and AIBN leads to formation of carbon center radicals that under aerobic condition causes formation of peroxyl radicals. This can be achieved in the absence of any metal catalyst. AAPH and AIBN can produce a steady state concentration of alkoxyl radicals for extended periods of time (26). These species are capable of modifying proteins, DNA, and membranes. Stadtman and coworkers (34) showed that 15 mM dosage of AAPH leads to complete inactivation of glutamine synthetase under physiological conditions. Further, it was shown that AAPH modifies histidine, tyrosine, methionine, and tryptophan residues (34). Palozza et al. have investigated the effect of fatty acid unsaturation on inhibition of AIBN- induced lipid peroxidation in hexane solutions, and β-carotene and α-tocopherol were shown to effectively suppress AIBN-induced MDA production (35).

5-ASA has been used as a therapeutic drug for inflammatory bowel disease. It is thought that chronically inflamed intestine is subjected to oxidative stress due to the presence of large amount of phagocytic leukocytes. Under certain inflamatory conditions, leukocytes can produce ROS that will damage intestinal muscosa cells (26,36–39). As stated above, 5-ASA is able to scavenge the free radicals in cardiomyocytes exposed to adri-

amycin that is used in the chemotherapy treatments (28).

Goncalves et al. investigated the antioxidant effects of 5-ASA against AAPH-induced peroxidation of phoshatidylcholine liposomes (27). A synergistic interaction between 5-ASA and α-tocopherol that significantly inhibits the oxidation of lipsosomes was suggested; however, 5-ASA alone did not prevent the oxidation (27).

EPR spin labeling methods are sensitive tools for determination of extent of protein oxidation, crosslinking, and lipid peroxidation. The motion of the protein specific spin label MAL-6, which covalently binds protein sulfhydryl groups of cysteine residues, is significantly decreased upon protein oxidation. This information can be deduced by measuring the relevant EPR parameter, the W/S ratio, which corresponds to the ratio of spectral amplitude of the $M_1 = +1$ lines low-field of the MAL-6 spin label. These lines indicate the weakly (W) and strongly (S) immobilized binding sites (8,32), with respect to motion of the spin label. Upon protein crosslinking, increased protein-protein interactions, or decreased segmental motion of the protein, the weakly immobilized binding sites will experience hindrance of their movement, and the W/S ratio of the spin label will correspondingly decrease (33).

We utilized EPR MAL-6 protein labeling to investigate the magnitude of protein modification in synaptosomal membranes treated with AAPH and AIBN. Oxidative modification of synaptosomal membrane proteins upon treatment with free radical initiators were observed. 5-ASA alone can prevent or modulate these changes. However, when very high concentrations of AAPH were used (10 mM), the 5-ASA at 20 μM was ineffective in scavenging the free radicals (data not shown). The results suggest that 5-ASA offers a great deal of protection against oxidative modification of proteins exposed to the aqueous phase. 5-ASA, being charged, presumably is able to directly intercept the alkoxyl radical present in the synaptosomal membrane without causing any additional changes to the protein structure.

Free radical reaction causes the oxidative modification of membrane bilayers (40,41) whose unsaturated chains are highly susceptible to free radical reactions. These processes result in changes of membrane structure and functionality. The use of 5-NS spin label can determine if there are changes in membrane fluidity by measuring the HWHH parameter of 5-NS label incorporated in the lipid bilayer (33,41,42). The position of the paramagnetic label is in this case closer to the hydrophilic surface; thus, the changes of the fluidity near the lipid-water interface can be monitored. In addition, based on changes in the EPR spectrum, one can determine if 5-ASA is able to partition into the hydrophobic membrane region. The HWHH reflects the rate of reorientation of the 5-NS spin label between parallel and perpendicular orientations with respect to the magnetic field direction. As the bilayer becomes more rigid, this reorientation rate decreases, and similar to chemical exchange phenomena, the HWHH decreases (33,41,42). Our results demonstrate a decreased membrane fluidity caused by 1 mM AIBN versus samples obtained from control animals, whereas pretreatment with 5-ASA (20 μM) significantly brings the HWHH values close to the control values. In addition, 50 μM HNE, the by-product of lipid peroxidation (40), similarly decreased membrane fluidity in gerbil cortical synaptosomes. Interestingly, 1 mM AAPH does not change membrane fluidity. This fact is likely attributed to the hydrophilic nature of the prooxidant, and thus its inability to penetrate the hydrophobic membrane bilayer. The mechanism and the effectiveness of free radical scavenging can be deduced by the chemical structure of the 5-ASA. Phenols can undergo proton removal or electron transfer reaction where phenol is converted to the corresponding phenoxyl radical. The stability of the given phenoxyl radical via resonance structure greatly determines the antioxidant capability of the given phenol derivative. 5-aminosalicylic acid with an electron donating amine group present para to the phenyl group on the ring offers such stability (43).

Our results show the potent nature of 5-ASA as an antioxidant in synaptosomal membrane in vitro. Since the synapse likely is involved early in neurodegeneration in AD (44), and due to the structural similarity of 5-ASA to other compounds that are able to cross the blood-brain barrier (BBB) (i.e., salicylic acid, aspirin), we believe 5-ASA should be able to cross the blood-brain barrier and can be further tested in experiments in vivo in models of neurodegenerative disorders associated with oxidative stress.

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