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# Inhibition by Aminosalicylates of Phosphatidic Acid Formation Induced by Superoxide, Calcium or Spermine in Enterocyte Mitochondria

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**ABSTRACT.** Inflammation is associated with oxidative stress and altered cellular calcium homeostasis. Our earlier studies have shown that, increased phosphatidic acid (PA) formation occurred in enterocyte mitochondria when exposed to superoxide, divalent metal ions or polyamines resulting in altered lipid composition. Since aminosalicylates are the drug of choice for gut inflammation, we have tested the effect of aminosalicylates on PA formation by enterocyte mitochondria. When stimulated by superoxide, $Ca^{2+}$ or spermine, phosphatidylethanolamine (PE) degradation and PA formation occurred in enterocyte mitochondria which can be inhibited by aminosalicylates. The inhibition was 50–60% at 0.5-mM concentration and at 1- or 2-mM final concentration, complete inhibition was observed. Both 5-aminosalicylate (5-ASA) and 4-aminosalicylate (4-ASA) showed similar effects. The stimulation of PA formation by calcium or spermine was not due to increased generation of superoxide by mitochondria which was confirmed by measurement of superoxide production by the mitochondria. These studies suggest that in addition to other cellular effects, aminosalicylates may prevent the enterocyte mitochondrial damage by inhibition of PA formation and PE degradation and alteration of mitochondrial lipid composition. BIOCHEM PHARMACOL **55**;9:1489–1495, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** aminosalicylates; enterocyte mitochondria; phospholipase D; oxidative stress

Inflammatory conditions are associated with oxidative stress and infiltrated phagocytes, especially, activated neutrophils are responsible for the generation of oxygen free radicals [1–4]. Mitochondria also form an important source of free radicals [5–7]. Oxidative stress is associated with changes in mitochondrial function. Normally $Ca^{2+}$ is sequestered in endoplasmic reticulum and a small amount is stored in the mitochondria [8]. Oxidative stress increases the cytosolic $Ca^{2+}$ level and this alters the mitochondrial $Ca^{2+}$ flux [9]. It is known that polyamines also influence the mitochondrial $Ca^{2+}$ flux in addition to their role in other cellular functions [10]. Structural alteration to mitochondria may be brought about by changes in the mitochondrial lipids and this may be due to the activation of certain mitochondria associated phospholipases. Our earlier work has shown that enterocyte mitochondria contain a PLD† which can be activated by superoxide, calcium or polyamines [11–13]. This PLD is unique in that, it does not catalyze transphosphatidylation reaction which has been shown for PLD from other sources and in this respect the enterocyte mitochondrial PLD resembles yeast PLD which also is unable to catalyze [14, 15]. The enterocyte PLD utilizes preferentially PE as substrate generating PA. Phosphatidic acid is not only an important intermediate in the synthesis of phospholipids and glycolipids but is also suggested to be an intracellular signaling molecule in eukaryotic cells [16–21]. It is rapidly produced in stimulated cells by the action of PLD.

5-ASA and 4-ASA are useful compounds for the treatment of chronic inflammatory bowel disease [22–24]. Their mode of action is not clear, but they have the ability to scavenge free radicals which is thought to be responsible for the inflammatory reactions. In the present study, we have investigated the enterocyte mitochondrial lipid alterations induced by exposure to free radicals, calcium or spermine and the effect of aminosalicylates on this lipid alterations.

## MATERIALS AND METHODS

### *Materials*

Various lipid standards, 5-amino salicylic acid, 4-amino salicylic acid, acetyl salicylic acid, spermine, X, XO, HEPES, fluorescamine and BSA were all obtained from Sigma Chemical Co. All other chemicals used were of analytical grade.

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† *Abbreviations:* 5-ASA, 5-aminosalicylic acid; 4-ASA, 4-aminosalicylic acid; MTT, [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]; PA, phosphatidic acid; PE, phosphatidylethanolamine; PLD, phospholipase D; X, xanthine; XO, xanthine oxidase.

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### *Preparation of Mitochondria*

Rats weighing 150–200 gm fasted overnight, were decapitated, the small intestine removed and washed with ice cold saline. Mitochondria were isolated from enterocytes as described by Masola and Evered [25]. Isolated mitochondrial fraction was suspended in EGTA free medium containing 250 mM of sucrose and 5 mM of HEPES pH 7.4 and stored in ice at protein concentration of 8–10 mg/ml. Protein was measured using BSA as standard [26].

### *Incubation of Mitochondria With X + XO, Calcium or Spermine and Lipid Analysis*

Mitochondria (1 mg of protein/mL), in suspension medium were incubated at 37° for 30 min with xanthine-xanthine oxidase (1 mM and 100 milliunits respectively), $Ca^{2+}$ (100 μM) or spermine (0.5 mM) separately (all final concentration). The effect of aminosalicylates were tested at 0.5, 1.0 and 2.0 mM (final concentration) on mitochondrial lipid alteration brought about by the above mentioned compounds. Control mitochondria were incubated for the same duration without addition of activators or salicylates. Following incubation, total lipids were extracted by Bligh and Dyer's method [27] and PA formed was separated by TLC and quantitated by phosphate estimation. Extracted lipids were spotted on silica gel G plates impregnated with 0.5 M of oxalic acid and separated using the solvent system chloroform-methanol-Con. HCl (85:134:0.5 v/v) [28]. PA spot corresponding to standard was identified by iodine exposure, scraped and eluted from the plates. PA was quantitated by phosphate estimation after acid digestion [29]. Our earlier studies using intestinal mitochondrial PLD have shown that this enzyme does not catalyze transphosphatidylation in the presence of alcohol [11–13] which is similar to recent reports of a yeast PLD unable to catalyze transphosphatidylation [14, 15]. Neutral lipids were separated on silica gel G plates using the solvent system hexane-diethyl ether-acetic acid (80:20:1 v/v). Spots were identified by iodine exposure, scraped and eluted. Cholesterol [30], diglycerides and triglycerides [31] were estimated as described. Free fatty acids were methylated and quantitated by gas chromatography after separation on a 5% EGSS-X column. Heptadecanoic acid was used as internal standard. Individual phospholipids were separated on silica gel H plates using the solvent system chloroform-methanol-acetic acid-water (25:15:4:2: v/v) [32] and quantitated by phosphate estimation after acid hydrolysis. Individual aminophospholipids were also quantitated after derivatisation with fluorescamine and separation on silica gel H plates impregnated with 3% magnesium acetate using the solvent system chloroform-methanol-$NH_4OH$-water (60:40:5:2 v/v) [33]. Eluted individual spots were quantitated using Shimadzu SF 5000 spectrofluorometer with excitation at 395 nm and emission at 468 nm.

### *Superoxide Measurement*

To perform the assay, following reagents were added in microtiter wells sequentially. The incubation mixtures contain 1.25 mM of MTT, either 1 mM of calcium or 1 mM of spermine (final concentration), mitochondria (60–100 μg) and phosphate buffer pH 7.4 in a total volume of 150 μL. The mixture was incubated for 30 min at room temperature (30°) and the reaction was stopped with the addition of 150 μL of DMSO, which also helps to dissolve the MTT formazan crystals formed. Plates were shaken for few minutes in an orbital shaker and were read on a microplate reader (Bio Rad Microtiter reader Model 450), using test wavelength of 570 nm and reference wavelength of 630 nm. Amount of superoxide generated was calculated using the molar extinction coefficient of MTT formazan $E_{570}$ of 17,000 $M^{-1}$ $cm^{-1}$ at pH 7.4 to 8.0.

### *Statistical Analysis*

Three separate estimations were carried out and results are presented giving the mean ± SEM. Mann–Whitney U test was done to compare the changes.

## RESULTS

We have recently reported that in rat intestinal mitochondria, oxygen free radicals, calcium or spermine can stimulate PLD-mediated hydrolysis of phosphatidylethanolamine resulting in the formation of PA. Fig. 1 shows the effect of exposure of enterocyte mitochondria to superoxide generated by X + XO on the PA formation and phosphatidylethanolamine depletion and the effect of 5-ASA and 4-ASA on this lipid alteration. As compared to control, exposure to superoxide increased the formation of PA (Fig. 1A) with a corresponding decrease in PE content (Fig. 1B) of phospholipids in mitochondria. Inclusion of aminosalicylate prevented this alteration of mitochondrial phospholipids brought about by superoxide. Aminosalicylates at 0.5 mM final concentration inhibited the PA formation approximately 50–60% whereas at 1.0- or 2.0-mM concentration, inhibition was complete. This was also reflected by the inhibition of PE degradation. Figure 2 shows the calcium stimulation of mitochondrial PA formation and PE degradation and the effect of aminosalicylate at different concentration inhibiting this process. A similar observation was seen with the effect of spermine on mitochondrial lipid alteration and the inhibitory effect of aminosalicylate on this process (Fig. 3).

To rule out the possibility of lipid changes due to increased superoxide generation by mitochondria in response to calcium or spermine and the superoxide scavenging effect of aminosalicylate in inhibiting these lipid alterations, superoxide formation by intestinal mitochondria in presence of calcium or spermine was measured by tetrazolium dye, MTT, reduction. As shown in Table 1, neither calcium nor spermine increased the formation of superoxide

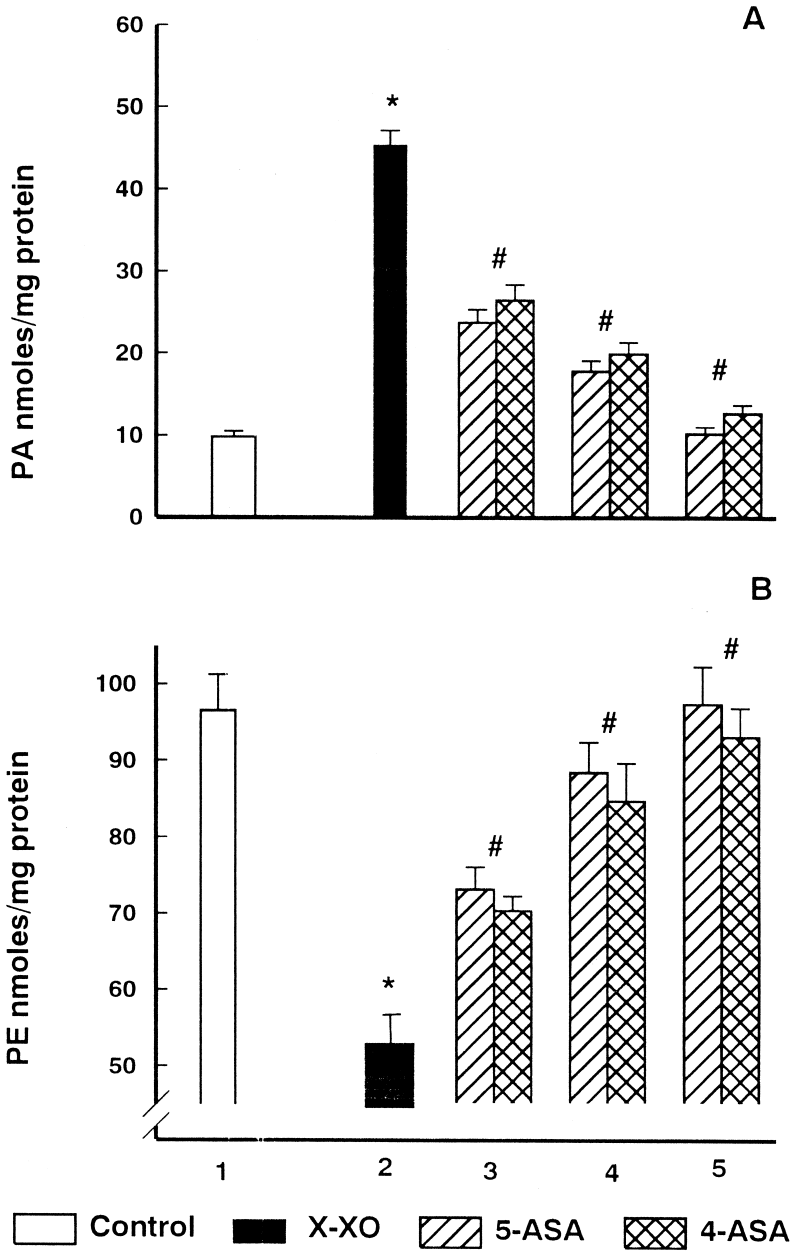


**FIG. 1. Inhibition of PA formation (A) and PE degradation (B) in rat enterocyte mitochondria by aminosalicylates after stimulation with X + XO system. The experimental details are described in the text. (1) Control incubated mitochondria; (2) mitochondria incubated with X + XO; (3) mitochondria incubated with X + XO in presence of 0.5 mM of 5-ASA or 4-ASA; (4) mitochondria incubated with X + XO in presence of 1 mM of 5-ASA or 4-ASA; (5) mitochondria incubated with X + XO in presence of 2 mM of 5-ASA or 4-ASA. Each value represents mean ± SEM of three separate estimations. *$P < 0.05$ compared to control. #$P < 0.05$ compared to mitochondria incubated with X + XO alone.**

by mitochondria. PA formation by the mitochondria was negligible in the absence of any of these stimulatory compounds (data not shown). It was also observed that in addition to aminosalicylates, acetylsalicylate (1 mM and 2 mM) also inhibited PA formation and PE degradation when stimulated by the above mentioned compounds (data not shown).

## DISCUSSION

Mitochondria are one of the sources of oxygen free radicals in the cell and it is estimated that 2–3% of total $O_2$ consumed in the cell is univalently reduced to superoxide which can also be converted to other free radicals [34]. Mitochondria also play an important role in cellular $Ca^{2+}$ homeostasis especially when the cytosolic $Ca^{2+}$ level is increased under certain stress conditions [35]. Mitochondria are responsible for the ATP generation and maintenance of cellular high energy compounds and have been recognised as a potential target of drug-induced toxicity [36, 37].

Aminosalicylates are commonly used drugs for the inflammatory conditions of the gut. Oxygen free radicals have been implicated in the pathogenesis of inflammatory conditions and aminosalicylates have been shown to scavenge free radicals [38]. 4-ASA is a weak scavenger of free radicals as compared to 5-ASA but is as effective clinically as 5-ASA [22, 39, 40]. Exposure of colon to 5-ASA prevents early surface epithelial cell loss as well as the subsequent increase in mucosal ornithine decarboxylase and thymidine incorporation in to mucosal DNA in response to sodium

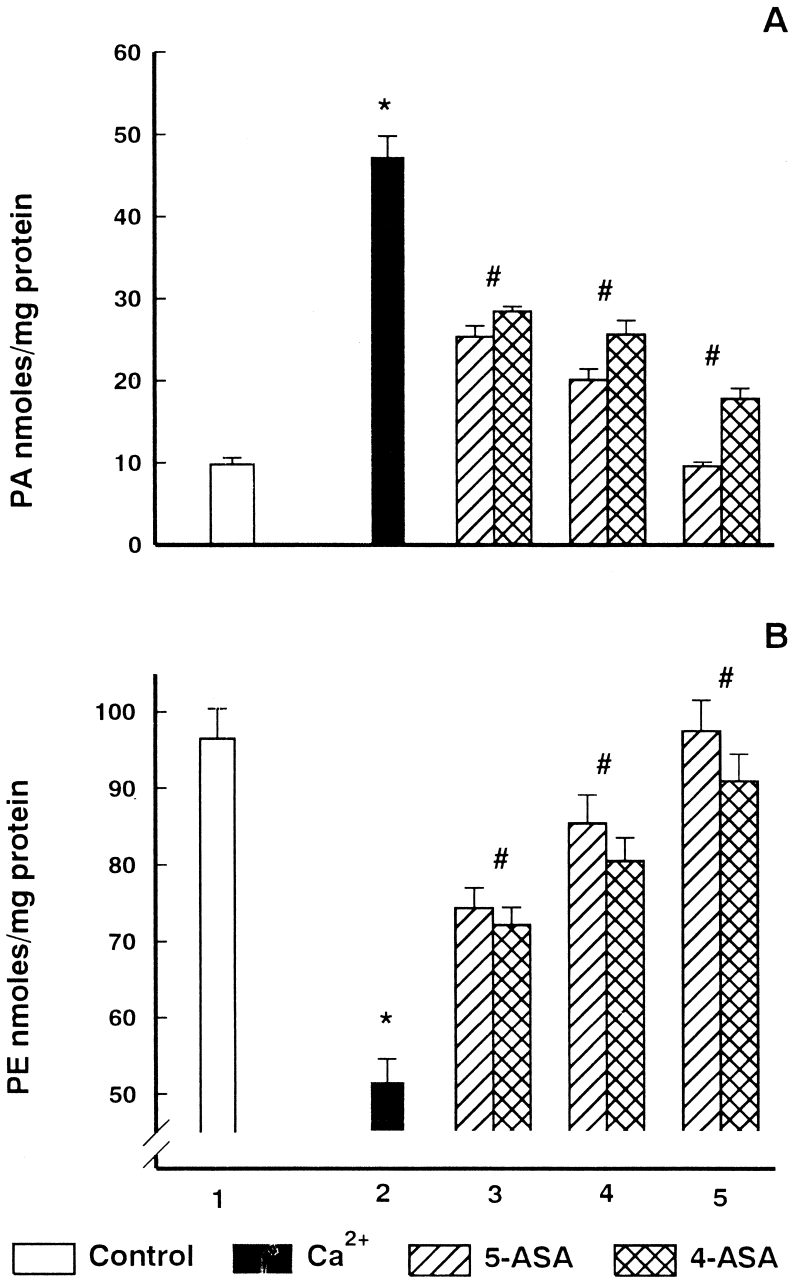


**FIG. 2. Inhibition of PA formation (A) and PE degradation in rat enterocyte mitochondria by aminosalicylates after stimulation with 100 μM of $Ca^{2+}$. The experimental details are described in the text. Details of the figure are as shown for Fig. 1. Each value represents mean ± SEM of three separate estimations. *$P < 0.05$ compared to control. #$P < 0.05$ compared to mitochondria incubated with 100 μM of $Ca^{2+}$ alone.**

deoxycholate. These actions of 5-ASA correlated with its ability to quench reactive oxygen species as measured by either SOD inhibitable chemiluminescence or by cyt.C reduction [41]. 5-ASA also suppressed superoxide production by X + XO system [42]. Although most of the actions of ASA are extracellular [43], some of the actions also may be intracellular since a significant amount of 5-ASA has been found within the cells. In addition, other important actions of 5-ASA such as inhibition of leukotriene B4 synthesis and antibody secretion, also have been demonstrated to be intracellular [44–46]. Free radicals may have various cellular targets and mitochondrial damage may be one among them. One possible mechanism by which mitochondria can be damaged is by changes in its structural composition, especially the alteration in their membrane lipid composition. Our earlier work has shown that, enterocyte mitochondria have an active PLD which can be stimulated by superoxide anion and various metal ions [11, 12]. This PLD preferentially hydrolyses PE resulting in the formation of PA. This lipid alteration may have an influence in the mitochondrial function.

In the present study, we have shown that the enterocyte mitochondrial PA formation stimulated by oxygen free radicals, $Ca^{2+}$ or polyamines, is inhibited by aminosalicylates. This was further corroborated by the alteration in the PE content of the mitochondria. Since aminosalicylates are known to scavenge free radicals, it is possible that the effect of salicylates on PA formation stimulated by X + XO may be due to scavenging of superoxide produced in this system. The fact that aminosalicylates were able to inhibit PA

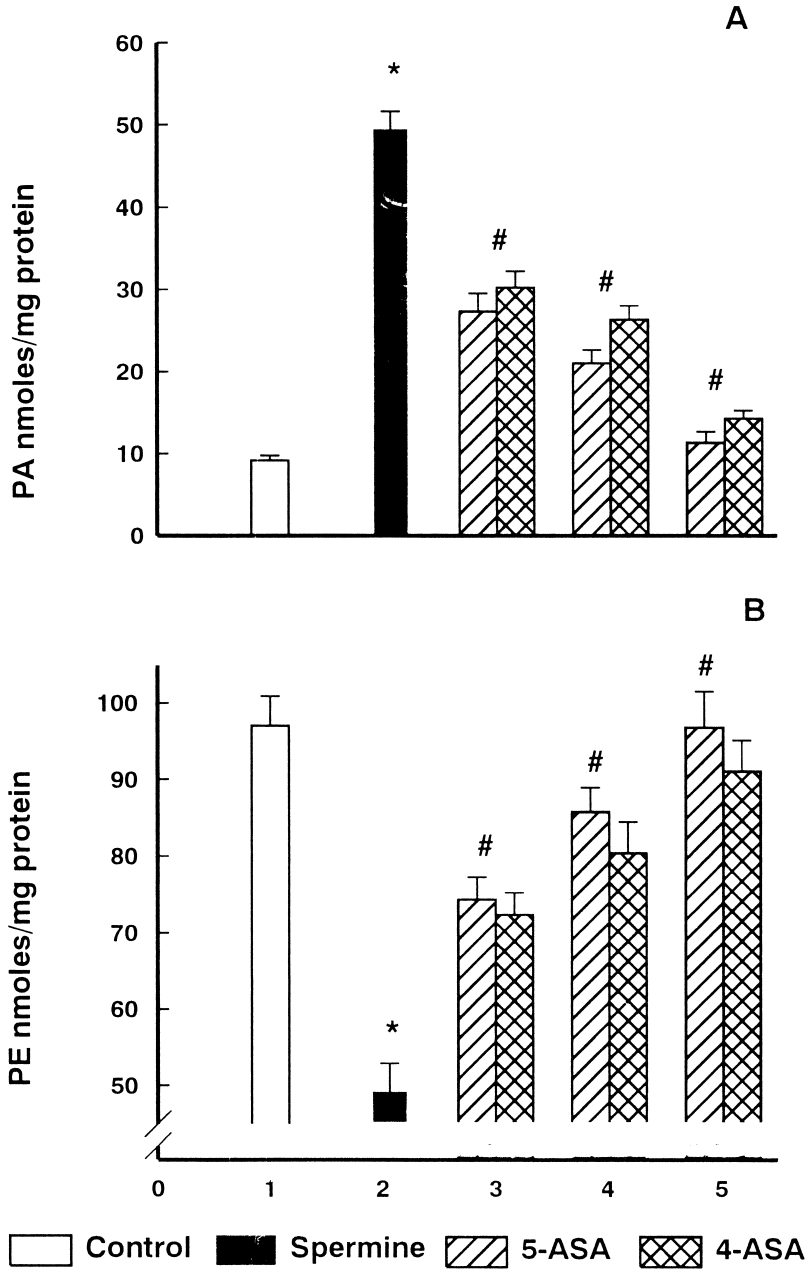


**FIG. 3. Inhibition of PA formation (A) and PE degradation (B) in rat enterocyte mitochondria by aminosalicylates after stimulation with 0.5 mM of spermine. The experimental details are described in the text. The details of the figure are as shown for Fig. 1. Each value represents mean ± SEM of three separate estimations. *$P < 0.05$ compared to control. #$P < 0.05$ compared to mitochondria incubated with 0.5 mM of spermine alone.**

**TABLE 1. Superoxide generation in control and calcium or spermine exposed enterocyte mitochondria**

| | Formazan formed (nmol) |
|---|---|
| Control mitochondria | 49.80 ± 2.70 |
| + calcium (0.1 mM) | 51.35 ± 2.00 |
| (1.0 mM) | 50.80 ± 2.30 |
| + spermine(0.5 mM) | 49.30 ± 2.30 |
| (1.0 mM) | 50.20 ± 2.80 |

Mitochondrial superoxide generation was measured as described in the text. Each value represents mean ± SEM of three separate estimations.

formation stimulated by $Ca^{2+}$ and spermine suggest that, the action of aminosalicylates is likely to be directly on the PLD enzyme. It was also observed that increased PA formation induced by calcium or spermine is not due to increased superoxide formation by mitochondria which was confirmed by detection of superoxide by tetrazolium dye reduction which showed no stimulation of superoxide by mitochondria in presence of calcium or spermine. It was shown earlier that phospholipase C activity is inhibited by acetyl salicylate [47]. To our knowledge, there is no report on the effect of salicylates on PLD activity. It is possible that aminosalicylates in addition to scavenging free radicals, during inflammation can also protect the epithelial cell mitochondria from damage

brought about by membrane lipid alteration due to phospholipase D activation.

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