Phospholipid Hydroxyalkenals
Biological and Chemical Properties of Specific Oxidized Lipids Present in Atherosclerotic Lesions

Henry F. Hoff, June O’Neil, Zhiping Wu, George Hoppe, Robert L. Salomon

Objective—Phosphatidylcholine hydroxyalkenals (PC-HAs) are a class of oxidized PCs derived from lipid peroxidation of arachidonate or linoleate at the sn-2 position to form terminal γ-hydroxy, α-, and β-unsaturated aldehydes. The aim of this study was to characterize some of their biological properties, ascertain the mechanism of their action, and assess whether they have in vivo relevance.

Methods and Results—Combinations of cell biological approaches with radiolabels, mass spectroscopy, and immunochromatography as well as immunohistochemical techniques were used to show that PC-HAs reduce the proteolytic degradation by mouse peritoneal macrophages (MPMs) of internalized macromolecules, such as maleylated bovine serum albumin, and that the activity of the lysosomal protease, cathepsin B, in MPMs form Michael adducts with MPM proteins and with N-acetylated cysteine in vitro form pyrrole adducts with MPM proteins and reduce the maturation of Rab5a, thereby impairing phagosome-lysosome fusion (maturation) in phagocytes; they are present unbound and as pyrrole adducts in human atherosclerotic lesions.

Conclusions—PC-HAs are present in vivo and possess multiple functions characteristic of oxidized LDL and 4-hydroxynonenal. (Arterioscler Thromb Vasc Biol. 2003;23:275-282.)

Key Words: oxidized phospholipids | macrophages | intracellular degradation | biochemical adducts | atherosclerotic lesions | Rab5

Oxidation has been demonstrated to play a major role in a number of human disorders, such as atherosclerosis and the degenerative disorders of aging. However, a more detailed understanding of the underlying mechanisms by which oxidative events affect these processes is still needed. Oxidation (ox) of LDL consists of a complex series of events leading to multiple changes in the composition of the lipid and protein constituents of LDL. One of the major modifications consists of the free radical–induced formation of hydroperoxides that subsequently undergo alkoxyl radical formation and β-scission to form a variety of products, such as reactive aldehydes. One of them, 4-hydroxynonenal (HNE) diffuses from oxLDL or oxidized cell membranes to form covalent adducts with cell proteins, inducing a loss of protein function, such as enzyme activity. HNE forms Michael adducts with side chains of lysines, histidines, and cysteines in proteins and pyrrole adducts with lysines. HNE also modifies LDL to induce enhanced recognition by macrophages.

Carboxyl-terminal analogues of HNE (methyl terminal) form during lipid peroxidation in both oxidized arachidonate and linoleate esterified to cholesterol or phosphoglycerides in phosphatidylcholine (PC). The likely mechanism of formation of 5-hydroxy-8-oxo-6-octenoic acid (HOOA) or its ester with PC (PC-HOOA) from arachidonate, and 9-hydroxy-12-oxo-10-dodecenoic acid (HODA) or its esters with PC (PC-HODA) from linoleate involves the partitioning of a family of peroxyeicosatetraenyl radicals generated by nonregioselective hydrogen atom abstraction from arachidonate or linoleate. β-Scission of an alkoxyl radical derived from dihydroperoxide could form two γ-hydroxy-α,β-unsaturated aldehydes such as HNE and an analogue still esterified to PC (see Figure 1), as was recently proposed. For every HNE molecule formed, a mirror image of esterified HNE is formed. Both PC-HODA and PC-HOOA have been produced by total unambiguous synthesis. Because they possess the γ-hydroxy-α,β-unsaturated terminal aldehyde characteristic of HNE, it is conceivable that PC-hydroxyalkenals (PC-HAs) would also form Michael adducts with the thiol group of cysteine in proteins, in addition to primary amino groups in lysyl residues and pyrrole adducts with lysines. Because the protein reactivity of these HNE-like termini might be similar to the highly reactive and toxic HNE itself, these oxidized lipids would be anticipated to exert important biological activities by forming protein-lipid adducts. Examples of potential altered functions
are the oxLDL-induced reduction in intracellular degradation of macromolecules previously internalized by receptor-mediated endocytosis or phagocytosis, concomitant with a reduction in lysosomal cathepsin B activity in mouse peritoneal macrophages (MPMs). Another is an oxLDL-induced reduction within phagocytes in the maturation of the fusion protein Rab5a in phagosomes, which could perturb endosome-phagosome maturation (fusion with lysosomes).

In this study, we sought to assess whether PC-HAs (1) mimic oxLDL, specifically HNE, in chemical reactivity, eg, formation of Michael additions with cysteines, and pyrrole adducts with lysines; (2) induce such functional changes in MPMs ascribed to oxLDL; and (3) are present in human atherosclerotic lesions. We now report that PC-HAs mimic HNE and oxLDL in chemical reactivity, in functional properties within MPMs, and in being present both unbound and as covalent adducts with proteins in lesions, thereby providing in vivo relevance to our observations in model systems.

Methods
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LDL Isolation and Oxidation
LDL was isolated by using a previously reported procedure, and subjected to lipid extraction with chloroform/methanol (1:2, vol/vol). Protein concentrations were determined by using bicinchoninic acid as described.

Degradation of Maleylated Bovine Serum Albumin by MPMs in Culture
Bovine serum albumin (BSA) was subjected to maleylation (mal) as described previously, and malBSA and LDL were labeled with NaO-2H by using a modified iodine monochloride procedure of McFarlane. Thioglycolate-elicited MPMs were harvested as previously reported and incubated with 125I-labeled malBSA for 21 hours, and cellular degradation was determined as reported. All results are reported as mean ± SD of triplicate determinations.

Cathepsin B Activity
Enzyme activity was measured in vitro according to the CLN procedure of Bajkowski and Frankfater, as used by us previously. Cathepsin B activity in macrophage extracts was also determined as described.

Western Blotting of Cell Lysates
MPM lysates were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) as described previously. Western blotting procedures included antibodies directed at anti-mouse cathepsin B, HNE-protein adducts that reflect mainly Michael adducts, and antibodies to HOOA-lysine or HODA-lysine pyrroles. Immunoasserted proteins were visualized by the ABC Vectastain technique.

Detection of Rab5a in Phagosomes
The rat retinal epithelial cell line RPE-J was grown as described and incubated for 5 hours at 40°C with PC-HA concurrently with magnetic latex beads; phagosomal and a nonphagosomal fractions were isolated with a magnet from cell homogenates and then subjected to Western blotting with anti-Rab5a.

Analysis of Michael Adducts of PC-HOOA With N-Acetylcysteine
Synthetic PC-HOOA and N-acetylcysteine (NAC) were mixed, and formation of a Michael adduct was determined by liquid chromatography with electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS).

Detection of PC-HOOA in Tissues and in oxLDL
Samples of human atherosclerotic lesions and oxLDL were subjected to lipid extraction, and their polar lipid fractions were then subjected to analysis by LC/ESI/MS/MS. Samples of standards were obtained by total unambiguous synthesis.

Immunohistochemical Detection In Vivo of Pyrrole Adducts of PC-HA and Lysine Side Chains in Proteins
Samples of human atherosclerotic plaques were subjected to immunohistochemistry with antibodies directed at HA-pyrrole adducts.

Results
Phosphatidylcholine Hydroxyalkenals
PC-HAs induce a reduction in MPM degradation of malBSA and in lysosomal cathepsin B activity. To establish whether PC-HA in oxLDL induced deficient processing in MPMs, we first assessed whether PC-HA reduced the degradation in MPMs of internalized 125I-labeled malBSA. Both PC-HODA and PC-HOOA reduced the ability of MPMs to degrade radiolabeled malBSA relative to control untreated cells or those pretreated with 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) by ≈30% to 40% (Figure 2a). By contrast, uptake was not affected (data not shown). We also determined the amount of active cathepsin B in intact cells by using the CLN assay and found that PC-HOOA or PC-HODA induced a significant reduction in enzyme activity in intact MPMs (Figure 2b), whereas unoxidized PLPC had no effect. When PC-HOOA or PC-HODA was incubated with cathepsin B under in vitro conditions, a significant reduction in activity was also found (data not shown). Cell protein synthesis as measured by the incorporation of [14]Cleucine into trichloroacetic acid precipitated radiolabel did not differ from that in untreated cells.
To evaluate whether the amount of cathepsin B mass was also affected by PC-HOOA, we ascertained by Western blotting any reduction in immunoreactivity of lysosomal cathepsin B in MPMs incubated with 10 μmol/L PC-HOOA for 24 hours. We found a clearly visible reduction in the enzyme mass, reflected by a decrease in immunostaining of the cathepsin B band in cells treated with PC-HOOA compared with cells treated with unoxidized PLPC (Figure 3a).

**Immunochemical Evidence for Michael Addition Formation of PC-HA With Protein Side Chains**

The reduction in cellular cathepsin B activity and, by inference, mass, induced by PC-HA in macrophages could also have been due to covalent adduct formation of cathepsin B with PC-HOOA or PC-HODA, such as Michael adducts. This was suggested by our result showing that Western blotting of extracts of cells treated with PC-HOOA with an antibody to HNE-Michael adducts\(^ {26}\) immunostained a band (Figure 3b) that comigrated with 1 that was immunopositive for cathepsin B (data not shown). HOOA-modified BSA also reacted with this antibody directed at HNE-Michael adducts. The reduced immunostaining could be due not only to a loss of immunoreactivity owing to modification of cathepsin B by adduct formation with the PC-HOOA but also to a reduction in the amount of enzyme present at steady state, itself a reflection of reduced synthesis and/or increased degradation.

**LC/ESI/MS/MS Analysis of Interactions Between PC-HA and NAC**

α,β-Unsaturated aldehydes are known to form Michael adducts with thiol residues on proteins\(^ {2,10,11}\) but such interactions between PC-HA and cysteines have yet to be demonstrated. To better examine the potential structures that might be formed by the interactions between PC-HOOA and target proteins, we subjected mixtures of isolated PC-HOOA and NAC to LC/ESI/MS/MS analysis. Analysis of PC-HOOA incubated with buffer alone at pH 7.4 resulted in the detection of a single, major peak with a retention time of 10.46 minutes (Figure 4A, top). After incubation of PC-HOOA with NAC at 37°C for 3 hours, the total ion chromatogram illustrated the appearance of a broadened peak with leading and trailing shoulders, consistent with the formation of additional species (Figure 4A, bottom). MS analysis of the shoulder region was consistent with hemiacetal formation and demonstrated the new appearance of ions with a mass-to-charge ratio (m/z) of 813, the anticipated molecular ion of the Michael adduct for the reaction of PC-HOOA with NAC. To further demonstrate that the adduct formed represents the Michael-addition product of PC-HOOA and NAC, we performed MS/MS analysis in both the negative- and positive-ion modes. The negative-ion MS/MS spectrum of daughters derived from m/z = 813 is illustrated in Figure 4B. The fragmentation pattern observed is consistent with the proposed structure shown (Figure 4B, insert). Likewise, the positive-ion mass spectra illustrated a predominant daughter-ion fragment at m/z = 184.2, consistent with loss of a phosphocholine moiety (Figure 34).
and insert). Collectively, these results suggest that 1 product of PC-HOOA with the nucleophilic cysteine thiol moiety of proteins may be the formation of a Michael adduct.

**Influence of PC-HA on Intracellular Trafficking**

One possible mechanism by which intracellular processing of internalized macromolecules might be perturbed is the inhibition of maturation (fusion) of early endosomes to (with) late endosomes by incubation of cells with PC-HA. Rab5a is a fusion protein believed to be critical for phagosome and possibly endosome maturation. Rab5a exists as an immature doublet of 25 kDa and as a mature doublet of 23 kDa after posttranslational modification, i.e., isoprenylation and proteolytic cleavage. We asked whether preincubation of a phagocytic cell, the rat retinal pigment epithelial cell line RPE-J with PC-HODA, would affect Rab5a maturation in membranes of phagosomes formed after uptake of magnetic latex beads. RPE cells have phagocytic properties critical for the diurnal shedding of outer segments of rods and cones in the retina. We recently showed that oxLDL perturbs the processing of outer segments after phagocytosis by RPE cells. Cells were allowed to phagocytose paramagnetic beads for 5 hours while being incubated with PC-HODA. The cells were then lysed and the lysate subjected to a magnetic field, permitting separation of the phagosomes containing the magnetic beads from the remaining cell lysate and cell membrane. Phagosomes from cells treated with PC-HODA demonstrated a shift from lower (mature) to higher (immature) molecular-weight bands relative to cells treated with PLPC (Figure 5a). By contrast, there was no shift in band position in the nonphagosomal fraction. In addition, the amount of nonphagosomal Rab5a was significantly reduced. These results suggest that maturation of Rab5a in phagosomes is impeded in cells treated with PC-HODA. 125I-malBSA degradation and cathepsin B activity were also reduced in these RPE cells after their incubation with PC-HODA, without affecting malBSA binding or uptake (data not shown). Collectively, these results suggest that an additional mechanism, possibly responsible for the poor degrada-

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**Figure 4.** PC-HOOA forms Michael additions in vitro with NAC. LC/ESI/MS/MS was performed on PC-HOOA alone and mixtures of PC-HOOA and NAC. (A) Single-ion monitoring (SIM) of PC-HOOA alone (top) and of the mixture (bottom), (B) daughter-ion fragments of the Michael adduct in the negative-ion mode, and (C) daughter-ion fragments of the mixture in the positive-ion mode. Daughter-ion fragment patterns from m/z=811.3 are consistent with the proposed structures drawn (inserts). Note that the hemiacetal rather than the linear form of the Michael adduct is drawn.

**Figure 5.** (a) PC-HODA perturbs the maturation of Rab5a in retinal pigment epithelial cells (RPE). Rat RPE-J cells were allowed to phagocytose paramagnetic beads in the presence of PC-HODA or PC-linoleate (PLPC). After a 4-hour incubation, cells were homogenized, separated into phagosomal and nonphagosomal fractions with a magnet, and analyzed for the presence of Rab5a by Western blotting as described in Methods. (b) PC-HOOA forms pyrrole adducts with multiple target proteins in macrophages. MPMs were incubated with 5 μmol/L PC-HOOA for 12 hours at 37°C. Lysates of cells were then subjected to the Western blotting procedure after SDS-PAGE of the lysates as described in Methods. Multiple bands with weights ranging from 19 to >100 kDa were immunopositive.
Pyrrole Adducts of PC-HAs With Lysines in Biological Samples

PC-HAs have been shown to form pyrrole adducts with lysyl residues of proteins.\textsuperscript{16} We detected such adducts previously in oxLDL and other plasma proteins by an ELISA that included an antibody directed at pyroles formed when unbound HOOA interacts with lysines on proteins.\textsuperscript{16} Reactivity to this antibody was appreciably higher when PC-HOOA–protein adducts were subjected to saponification or digestion with phospholipase A\textsubscript{2}.\textsuperscript{16} We asked whether such adducts were also formed with cellular proteins after incubation of macrophages with 5 \textmu mol/L PC-HOOA for 12 hours. Multiple bands were immunostained in the molecular-weight range of 19 to \textasciitilde150 kDa on Western blots of cell lysates (Figure 5b). A “ladder” of polymerized proteins was seen when the HOOA-BSA pyrrole standard was immunostained (data not shown), indicating protein cross-linking by HOOA, analogous to the protein cross-links induced by HNE.\textsuperscript{29}

Because of the presumed chemical reactivity of PC-HA, we anticipated that pyrrole adducts with lysines on proteins would also exist in atherosclerotic plaques. To investigate this possibility, we used immunohistochemical techniques to localize epitopes that identify oxidative adducts containing pyroles in human atherosclerotic lesions. HOOA and HODA pyroles displayed identical localization patterns, so that only 1 will be described here. The predominant localization pattern in both early and more advanced human atherosclerotic lesions was to macrophages found surrounding the necrotic core and in the shoulder regions (Figure 6a). Macrophages were identified by immunostaining for CD68 (Figure 6b). However, not all CD68-positive cells were immunopositive for HOOA-pyrroles. Another separate localization pattern was to connective-tissue elements, presumably collagen bands, in the necrotic core (Figure 6c). This pattern was similar to 1 that showed localization of accumulated LDL (apoB-100) (Figure 6d). Controls, which included immunoblotting the PC-HA–lysine pyrrole antibody with HOOA-BSA or HODA-BSA or substituting nonimmune IgG in place of the anti-HOOA-pyrrole or anti–HODA-pyrrole, removed the aforementioned localization patterns (data not shown).

Unbound PC-HOOA Is Present in Human Atherosclerotic Lesions and in oxLDL

To assess whether PC-HAs have in vivo relevance, we asked whether we could identify PC-HOOA in human atherosclerotic lesions or in ox-LDL by LC/ESI/MS/MS and an authentic PC-HOOA standard prepared by unambiguous synthesis.\textsuperscript{17} We first subjected the PC-HOOA synthetic standard to LC/ESI/MS/MS in the negative-ion mode, samples of human atherosclerotic plaques, the oxLDL demonstrated a peak that coeluted with that of the synthetic standard (Figure 7c). Similarly, when samples of LDL oxidized with Cu\textsuperscript{2+} ions and unoxidized LDL were subjected to the same extraction and purification procedure as plaques, the oxLDL demonstrated a peak that coeluted with that of the synthetic standard. Furthermore, the peak for oxLDL was about 1 order of magnitude higher than the small peak for unoxidized LDL (Figure 7d). Thus, both human atherosclerotic lesions and ox-LDL contain a PC oxidation product with identical parent ions, characteristic...
daughter ions, and a retention time of authentic PC-HOOA, providing direct evidence that this /H9251, /H9252-unsaturated aldehyde is present in an in vivo setting.

**Discussion**

In this study, we found that PC-HA mimicked oxLDL and HNE in inducing a reduction in the ability of MPMs to degrade macromolecules, such as malBSA, which had been internalized by scavenger receptor–mediated endocytosis,23 in parallel with a reduction in the cellular cathepsin B level. Previously we showed that oxLDL reduced the intracellular degradation of oxLDL and aggregated LDL in MPMs concurrently with a reduction in cathepsin B, but not cathepsin D13, suggesting a causal relationship. These results were also obtained with a polar lipid fraction isolated from oxPC (O’Neil and Hoff, unpublished observations), suggesting that oxPCs in the outer shell of oxLDL were primarily responsible for these changes. We found that cathepsin B can be inactivated in vitro by incubation with oxLDL,8,9 HNE,7 or HNE-modified LDL.9 In a subsequent study with MS techniques, we found that such inactivation of cathepsin B was caused by Michael-adduct formation between HNE and a cysteine and a histidine in the active-site pocket of the enzyme.7 Given that PC-HAs have the same reactive functionality as HNE,16 it is possible that they may react in a parallel fashion. Our MS and immunochemical data collectively suggest such a mechanism, because Michael-adduct formation occurred between PC-HA and cathepsin B.

We also showed that PC-HA inhibited the posttranslational modification of Rab5a in phagosomes of RPE cells, an event that could have been responsible for reduced maturation (fusion) of phagosomes—early endosomes to form phagolysosomes—late endosomes,20 thereby reducing the processing of any macromolecules contained within these phagosome or endosomes. Because prenylation occurs at cysteine residues of proteins,20 Michael-adduct formation of PC-HA with cysteines would be expected to inhibit this step, which is critical for membrane fusion events and subsequent intracellular trafficking.20 It will be of interest in future studies to assess whether Rab5a forms Michael adducts with PC-HA within cultured macrophages.

PC-HAs were shown previously to form pyrrole adducts with lysyl residues on proteins.16 Indeed, HODA-derived pyrrole-containing modifications of human plasma proteins were found in subjects with angiographically documented cardiovascular disease, at levels that were significantly higher than in age-matched control subjects without cardiovascular disease.12 We show in this current study with Western blotting techniques that multiple proteins, yet to be identified, contained such pyrrole adducts after incubation of macrophages with PC-HOOA at a concentration of only 5 μmol/L. Although, strictly speaking, we cannot distinguish whether the HOOA-pyrrole in cellular protein is derived from protein adducts formed with free HOOA or HOOA esterified to PC or to cholesterol, it is likely derived from PC-HOOA, because they were the only form added to the cells, and untreated cells failed to show immunopositive bands (data not shown).

We also detected unbound PC-HA by MS in human atherosclerotic lesions and in Cu2+-oxidized LDL. Authentic PC-HOOA was subjected to LC/ESI/MS/MS in the positive- (A) and negative- (B) ion modes. Assignment of multiple characteristic ions is consistent with the proposed structure shown in insert. (C) LC/ESI/MS/MS in the positive-ion mode of a phospholipid fraction derived from extracts of human atherosclerotic lesions. MRM transitions from m/z=650 to 184 were monitored. (D) LC/ESI/MS/MS in the positive-ion mode of PC-HOOA in oxLDL (upper scan) and LDL (lower scan). MRM transitions (parent ion at m/z=650, daughter ion at m/z=184) were monitored.

**Figure 7.** Unbound PC-HOOA is detected in human atherosclerotic lesions and in Cu2+-oxidized LDL. Authentic PC-HOOA was subjected to LC/ESI/MS/MS in the positive- (A) and negative- (B) ion modes. Assignment of multiple characteristic ions is consistent with the proposed structure shown in insert. (C) LC/ESI/MS/MS in the positive-ion mode of a phospholipid fraction derived from extracts of human atherosclerotic lesions. MRM transitions from m/z=650 to 184 were monitored. (D) LC/ESI/MS/MS in the positive-ion mode of PC-HOOA in oxLDL (upper scan) and LDL (lower scan). MRM transitions (parent ion at m/z=650, daughter ion at m/z=184) were monitored.
Using immunohistochemical techniques, we also detected HA-pyrrole adducts in human atherosclerotic lesions. They presumably represent adducts formed with PC-HA but after removal of the PC “backbone,” possibly by a phospholipase A₂. As such, we cannot rule out their possible derivation from free HA or HA esterified to cholesterol. Because of the low immunoreactivity of such esters to this antibody, it is likely that a phospholipase A₂ (for PC-HA) or a cholesterol hydrolase (for cholesterol-HA) liberated the HODA-pyrrole. The localization pattern of these adducts to macrophages in lesions is similar to the distribution of several oxidation epitopes, such as reduced HNE-lysine adducts, in lesions from Watanabe heritable hyperlipidemic rabbits or those from apoE-null mice. The localization of these pyrrole adducts along large bands of connective tissue, presumed to be collagen fibers, would be anticipated, given the high content of lysines in collagen, and would indicate oxidative damage to these proteins, analogous to pyrrole adducts of HNE with lysines in collagen in human lesions.

We had also shown recently that oxPC, specifically a class of oxPCs with terminal α,β-unsaturated aldehydes or carbonyls, including PC-HA, functioned as ligands for CD36. It is possible that when macrophages were incubated in this study with PC-HOOA, internalization of the PC-HOOA may have been mediated by CD36, illustrating another important biological role of PC-HA. The covalent association of phosphorus with apoB-100 suggests the possibility that oxidized forms of PC were attached to apoB-100 in oxLDL, possibly Michael adducts. Schiff-base adducts formed between proteins such as BSA and oxovaleryl-PC, and reduced forms of these adducts were reported to function as a ligand for the CD36 scavenger receptor.

Subbanagounder et al recently quantified PC-HOOA in minimally modified/ox LDL and found that it represents about 2% of the oxPAPC in this form of oxLDL. They also described several proinflammatory properties of PC-HOOA, such as the ability to activate cultured endothelial cells, leading to a 2-fold increase in monocyte binding, and a dose-dependent increase in the synthesis of the chemokines, monocyctic chemotactic protein-1 and interleukin-8. In addition, they found that PC-HOOA inhibited the lipopolysaccharide-induced expression of the adhesion molecule E-selectin. The identity of the receptor on endothelial cells that mediates these functional properties still needs to be determined.

In summary, we have provided evidence suggesting that the PC-HA class of oxPCs is present in vivo, either in unbound or bound forms, and that they inhibit a key function of macrophages, namely, the processing of internalized material, possibly owing to its chemical reactivity. It is still uncertain whether the parallel reduction in cathepsin B activity or the documented reduction in the maturation of the fusion protein Rab5a, signifying reduced endosome-phagosome fusion with lysosomes, is responsible for this processing deficiency. Additional studies will be needed to evaluate the relative contribution of each of these mechanisms.

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References


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