



OXIDIZED [1-¹⁴C] LINOLEIC ACID AND HYDROGEN PEROXIDE TONE MIGHT PROMOTE ANTI-ATHEROGENIC ACTION AND PREVENT METABOLIC DISEASES

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ABSTRACT

Oxidized low density lipoprotein (Ox-LDL) and its various forms are responsible for development of proatherogenic action in in vitro cell culture system. Reactive oxygen species (ROS) can produce detrimental effects that include atherosclerosis. However, mild oxidized products could also impose antiatherogenic action. Present study suggests that HepG2 cells treated with [1-¹⁴C] Linoleic acid formed oxidized linoleic acid (13-hydroperoxy-9,11-octadecadienoic acid) [13-HPODE] and hydrogen peroxide [H₂O₂]. In addition to that cells exposed to oxidized [1-¹⁴C] HPODE showed peroxisomal degradation of oxidized [1-¹⁴C] HPODE to H₂O₂ which might be a lipoxygenase enzymatic reaction. Azelaic acid which is lipid peroxidation-derived lipophilic dicarboxylic acid is also formed during the reaction. The generation of this compound can induce anti-atherosclerotic effect. Similarly, in absence of HepG2 cells [1-¹⁴C] linoleic acid and [1-¹⁴C] HPODE independently formed least oxidation. Ox-LDL treatment to HepG2 cells at 0-4 h showed increase in H₂O₂ generation. Ox-LDL alone showed rise in lipid peroxide and H₂O₂ content at 1h. A cellular change was also noticeable effect of Ox-LDL and HPODE treatment.

KEYWORDS: *Linoleic acid; Lipoxygenase; 13-hydroperoxy-9,11-octadecadienoic acid.*



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INTRODUCTION

Large number of concluding evidences has suggested that Ox-LDL (Oxidized low density lipoprotein) plays significant pathological role in atherosclerosis¹⁻³. The pro-atherogenic action of Ox-LDL is attributed for its increased uptake of Ox-LDL by macrophages by scavenger receptors^{2,4}. Chandrakala et.al.⁵ have also reported that Ox-LDL and oxidized lipids of ischemic cells has pivotal action in inducing brain natriuretic peptide (BNP) and monocyte chemoattractant protein-1 (MCP-1) gene which are early biomarkers of heart failure⁵. Number of cell surface receptors was identified as putative receptors for Ox-LDL. It is known that theoretically native LDL circulating in plasma contains unmodified apoB-100 and no associated peroxides or aldehydes and it is rich in antioxidants and polyunsaturated fatty acids (PUFA). The PUFA or antioxidants are lost from LDL when LDL is "seeded"¹. However, when oxidation takes place at minimal level the particle represents it is minimally Ox-LDL or mm-LDL. In that form the particle that has intact ApoB-100 with minor protein damage or modification but phospholipids gets enormously affected⁶⁻⁷. Similarly, McCarthy et.al.⁸ reported that conjugated linoleic acid (CLA) can induce expression of Cholesterol Scavenger receptor ABCG1, cholesterol metabolizing enzymes CYP7B1 and PGC1 α target gene CPT1 α which is important in atherosclerosis. Their studies suggest that CLA can reduce atherosclerotic lesions, through the regulation of these genes involved in inhibiting the foam cell formation^{8,5}. Very few evidences are available to report that cells internalize Ox-LDL by any pathway other than the LDL receptor-mediated pathway however, these particles get more oxidized if they are subjected to oxidation in vitro. Steinbrecher et al.⁹ reported that in vivo clearance of Ox-LDL depends on the extent of particle oxidation. This suggests that components of LDL by mild oxidation are directly taken up by LDL receptor or it results to more extensive oxidation and increased uptake by the liver scavenger receptor. These particles which are devoid of PUFA, monounsaturated fatty acid (MUFA) and antioxidant are efficiently cleared from plasma by the liver, even though they are formed in plasma components. Ox-LDL also affects the monocyte differentiation into macrophages and it also induces the expression of receptors for its uptake¹. Staprans et al.¹⁰ showed in rabbit consuming oxidized (heated) fat is absorbed, incorporated into lipoproteins secreted by liver, and enhances the in vitro oxidizability of isolated LDL. In contrast to proatherogenic effect of oxidized lipids there could be potent anti-atherogenic effect of peroxidized lipids as it can induce synthesis of nitric oxide which shows vasoactive properties¹¹. Oxidized lipids can also elicit "antioxidant-antiatherogenic responses from cells for example it can induce manganese superoxide dismutase (Mn-SOD), heme oxygenase (HO1), catalase (CAT) and glutathione (GSH)¹. Meilhac et al.¹³ have also reported an increase in CAT gene in RFASMC when cells are treated with 13-HPODE (13-hydroperoxy-9,11-octadecadienoic acid), 13-HODE, MO-LDL, or H₂O₂. In spite of detrimental effects of oxidized fats (OF) in human health one such group have reported that oxidized fats (OF) can inhibit proatherogenic effect, by reducing monocyte recruitment and SMC proliferation

and migration. The OF can activate PPAR α in liver, which can cause lower lipid, TAG, cholesterol and VLDL levels in plasma and vasculature of rats. It has also been reported that feeding a diet rich in 13-HPODE can strongly reduce TAG levels in plasma that takes place by PPAR α -dependent pathway¹². Meilhac et al.¹³ have demonstrated that in smooth muscle cells (SMC) oxidized linoleic acid [13-HPODE] could increase intracellular H₂O₂, mediate cytotoxic effects. Based on these studies that are done so far the hypothesis in present studies is that Ox-LDL and oxidized products of linoleic acid such as 13-HPODE, H₂O₂ in minimal amount can prove to be beneficial to cells and may be antiatherogenic.

MATERIALS AND METHODS

HepG2 Cell Culture

HepG2 cells (American Type Culture Collection [ATCC], Manassas, VA) were routinely cultured in advanced DMEM (Invitrogen, Carlsbad, CA), supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) and were maintained at 37°C in an atmosphere containing 5% CO₂. For experiments the cells were seeded in 6-well dishes and before any experiment, cells were placed in their corresponding serum-free medium for 8-16h.

Isolation of LDL, preparation of minimally oxidized LDL and treatment of HepG2 cells with Ox-LDL

LDL (d 1.019-1.063) was isolated by ultracentrifugation, using TL-100 tabletop ultracentrifuge from collected blood of healthy donar, the LDL was then dialyzed against PBS, pH 7.4, for 6h. MO-LDL was prepared by adding 5 μ m CuSO₄ to LDL solution in PBS at 37°C and conjugated dienes that were formed were monitored at optical density (OD) of 234 nm for about 1h. The oxidation was halted by adding 10 μ m EDTA. The MO-LDL formed was then used for treating HepG2 cells. HepG2 cells were treated with Ox-LDL for 0,5,30 mins, 1, 2 and 4h period and cellular change were detected microscopically. The second experimental set was designed with and without HepG2 cells and Ox-LDL.^{4,31}

Preparation of 13-HPODE and treatment of HepG2 cells with 13-HPODE

Stock linoleic acid (C18:2) was prepared in absolute ethanol and it was then oxidized to 13-HPODE with soybean lipoxygenase (100U/mL) at 37°C for 1h. The 13-HPODE formed was monitored spectrophotometrically by scanning the absorption between 200 and 300 nm (model DB 3500, SLM-AMINCO; Spectronic Instruments, Rochester, NY) using PBS as reference. A conversion of linoleic acid to 13-HPODE is observed as an increase in absorbance at 234 nm.^{4,31}

Uptake and incorporation of [¹⁻¹⁴C] Linoleic acid into cellular lipids

HepG2 cells were seeded in 6-well plates and were grown until reached 90% confluence. Studies were performed in absence of serum by using 25 μ m concentrations (5,000 dpm/nmol) of labeled solution of linoleic acid. After different times of incubation, the medium was removed and cells were washed with PBS.

The cells were scraped into 2ml of 0.05% deoxycholate-containing PBS, and radioactivity was determined in 100 μ l of cell lysate. Results are expressed as a percentage of total radioactivity.³¹

N-Benzoyl LMB method for Peroxide Detection

The N-Benzoyl LMB method is used to measure the amount of free lipid peroxides present in system. It is a common method that measures peroxides such as H₂O₂ and other lipid peroxides. About 25 μ m/L of 13-HPODE was incubated with cells at 37°C for 1h. After incubation, 40 μ L of sample was put in each well of 96-well microtiter plate. The plate was incubated with 100 μ L of N-Benzoyl LMB color reagent (5mg LMB in dimethylformamide, 0.05mol/L K-phosphate buffer, pH 5.0, 1.4 g Triton X-100, 5.5 mg hemoglobin). After 5 min's at RT sample was read at 660 nm in a microtiter plate reader.

RESULTS

Uptake and incorporation of [1-¹⁴C] Linoleic acid and oxidized [1-¹⁴C] HPODE into cellular lipids by HepG2 cells

It is previously reported that, reactive oxygen metabolites react spontaneously with cellular lipids and can oxidatively modify fatty acids. Linoleic acid (LA) is a doubly unsaturated fatty acid, is found in plant glycosides and an essential fatty acid found in mammalian nutrition. Linoleic acid is most abundant PUFA present in the plasma and in LDL, and 13-HPODE is the oxidized form of linoleic acid produced by lipoxygenases¹³. HepG2 cells might possess 12-lipoxygenase, which under certain circumstances converts linoleic acid to its oxidized form. To determine

whether HepG2 cells convert [1-¹⁴C] Linoleic acid to 13-HPODE we incubated cells with [1-¹⁴C] Linoleic acid for 3h. We observed substantial increase in oxidized form of 13-HPODE at 3h seen as 1203.67 \pm 61.65 as compared to [1-¹⁴C] Linoleic acid without cells showed 552.33 \pm 37.09 which indicates that 12-lipoxygenase might be involved in its conversion (Figure 1A). However, at 1h time [1-¹⁴C] Linoleic acid did not form oxidized products in absence of cells. Figure 1A shows that oxidized [1-¹⁴C] HPODE at 4h in presence of HepG2 cells can bring peroxisomal degradation the values seen are 2393 \pm 39.95 however, in absence of cells oxidized [1-¹⁴C] HPODE at 2h showed 1078 \pm 59.5 which indicates least degradation of [1-¹⁴C] HPODE. This suggests that intracellular generation of H₂O₂ might be one such mechanism by which H₂O₂ can activate catalase.

Figure 1 (A)

Uptake and incorporation of [1-¹⁴C] Linoleic acid and oxidized [1-¹⁴C] HPODE into cellular lipids by HepG2 cells.

The experimental set up included treatment of Hep G2 cells with [1-¹⁴C] Linoleic acid and oxidized [1-¹⁴C] HPODE for 1-4h (Figure 1A respectively). HepG2 cells showed increase in % total radioactivity for [1-¹⁴C] Linoleic acid (3h) and oxidized [1-¹⁴C] HPODE (4h) when HepG2 cells are exposed to these compounds. However, [1-¹⁴C] Linoleic acid (1h) and oxidized [1-¹⁴C] HPODE (2h) in absence of cells showed least change (Figure.1A). Figure 1B indicates that HepG2 cells treated with HPODE show some morphological alteration which indicates that it might be generating H₂O₂

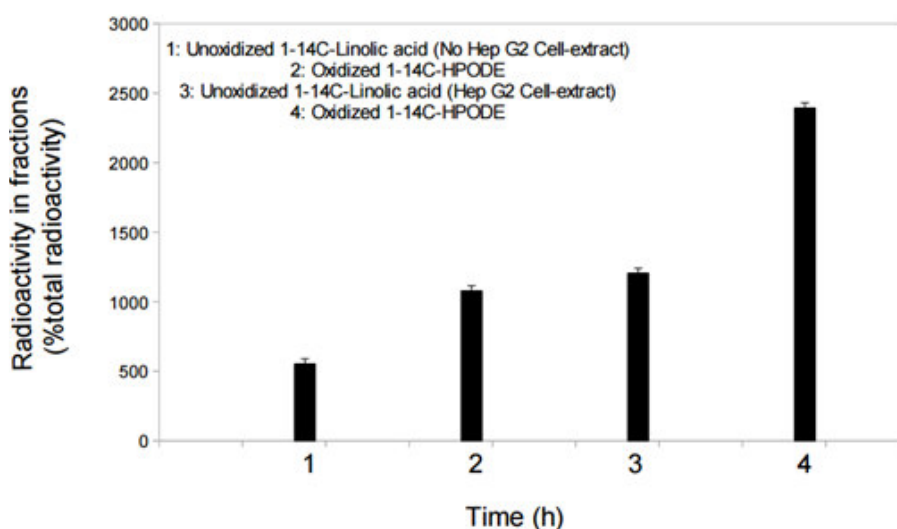


Figure 1A

Radioactivity Studies to detect conversion of 1-14C-Linolic acid to 13-HPODE and 1-14C-HPODE to H₂O₂ in prescence and absence of HepG2 cells

1. Unoxidized 1-14C-Linoleic acid (No HepG2 Cell-extract) (Lipoxygenase Absent)- 1 hour
2. Oxidized 1-14C-HPODE (No HepG2 Cell-extract) (Lipoxygenase Absent) – 2 hour
3. Unoxidized 1-14C-Linoleic acid (HepG2 Cell-extract) (Lipoxygenase Present)-3 hour
Significant Increase
4. Oxidized 1-14C-HPODE (HepG2 Cell-extract) (Lipoxygenase Present) – 4 hour
Significant Increase

Figure 1 (B)

HPODE Treatment of HepG2 cells

In Figure 1B depicts cells treated with HPODE for 0-4h showing alteration in morphology.

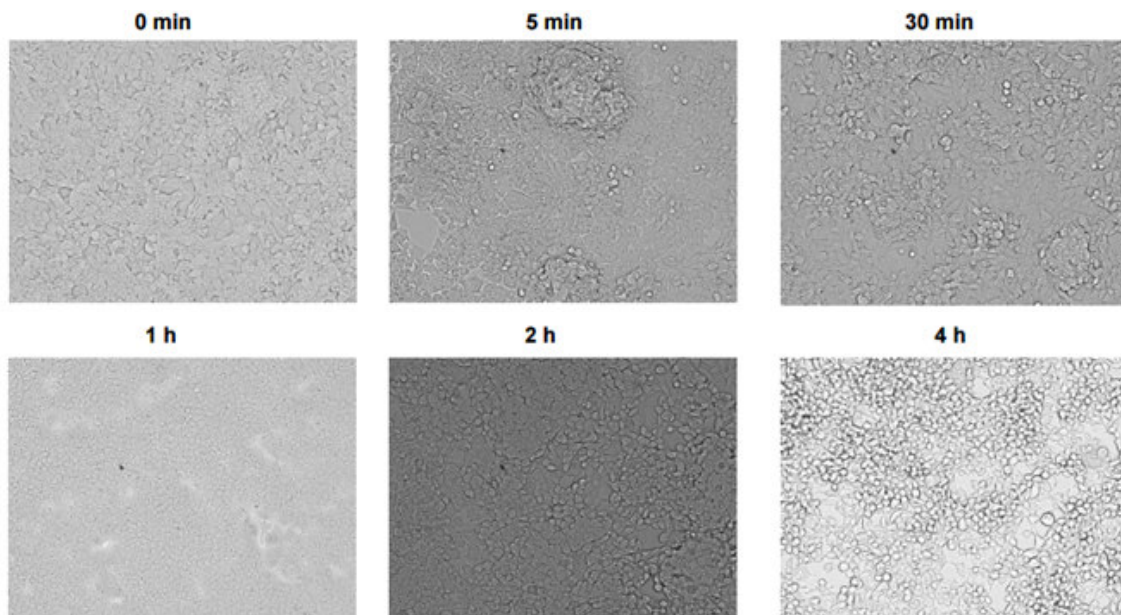


Figure 1B
HPODE Treatment of HepG2 Cells

Uptake and incorporation of oxidized LDL into cellular lipids by HepG2 cells

Ox-LDL is known to play a central role in atherosclerosis. When LDL undergoes mild oxidation (MO-LDL), its lipid peroxide content increases.¹³ Results presented in Figure 2A show that Ox-LDL in presence of HepG2 cells at 0-4h can increase the generation of H₂O₂ (2-4h). HepG2 cells exposed to Ox-LDL showed

significant increase at 0, 1, 2 and 4h seen as 0.21±0.01, 0.21±0.03, 0.23±0.02, 0.22±0.01 as compared to Ox-LDL without HepG2 cells shown at 0, 1, 2, 4h seen as 0.22±0.03, 0.31±0.02, 0.16±0.02 and 0.13±0.01 (Figure 2A). Figure 2B show morphological change in HepG2 cells from 0-4h which signifies that as time of incubation increases the cells might be accumulating more H₂O₂.

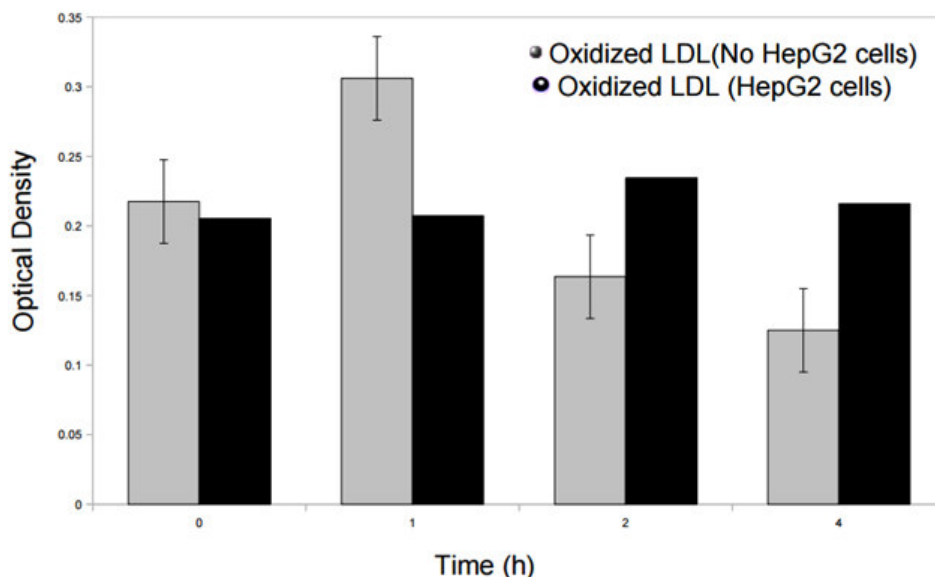


Figure 2A
Oxidized LDL in presence and absence of HepG2 cells

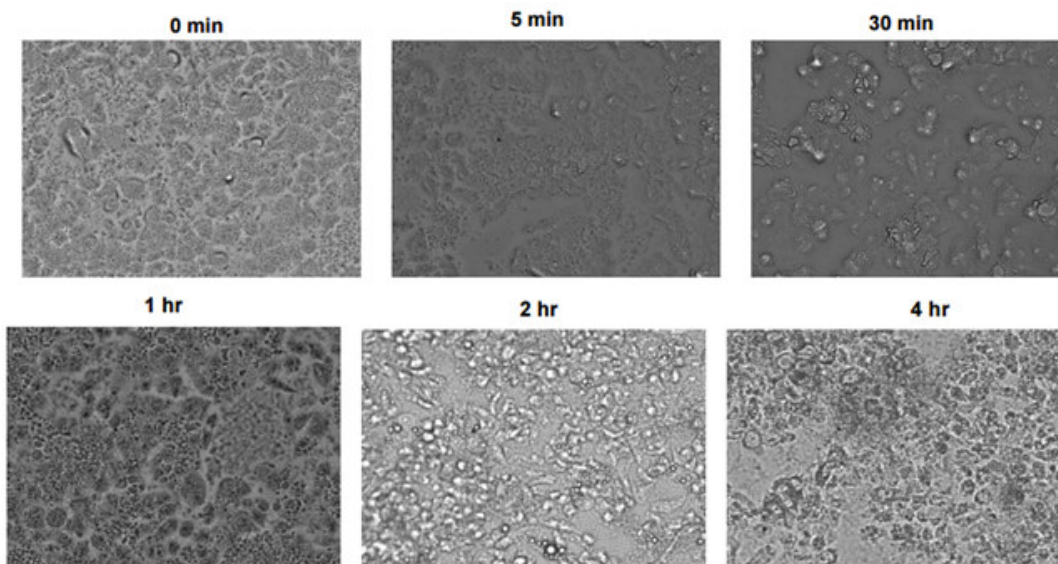
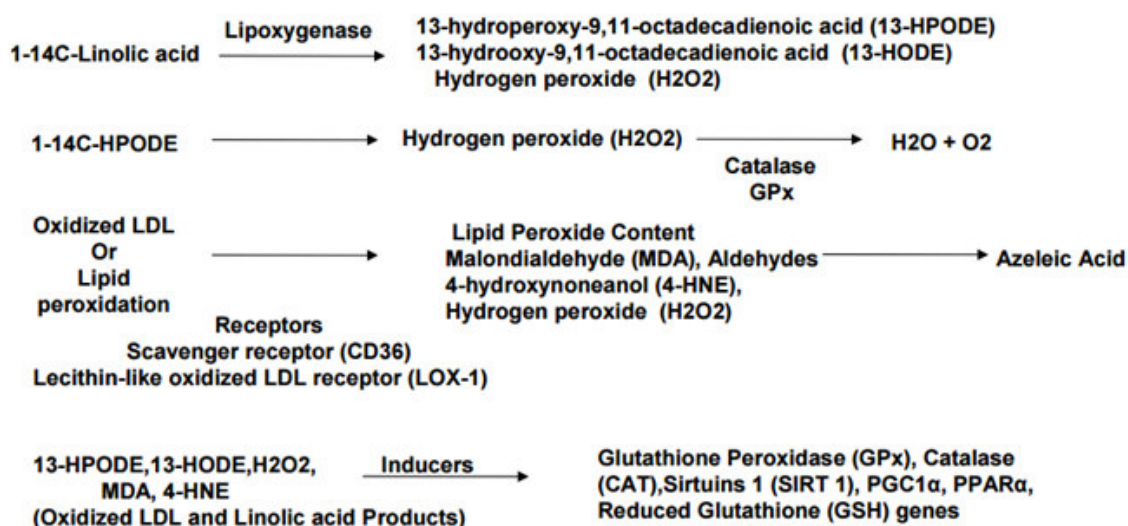


Figure 2B
Oxidized LDL Treated HepG2 cells

Schematic representation of mechanism of action of lipid peroxidation



DISCUSSION

The study demonstrates that HepG2 cells exposed to [1-¹⁴C] Linoleic acid is converted to oxidized form that is it forms 13-HPODE, however in absence of HepG2 cells there is little change which suggest that cells contains lipoxygenase enzyme system that might bring about oxidation reaction. Intracellular generation of H₂O₂ can take place from oxidized linoleic acid if cells are exposed for prolonged period of time. The 13-HPODE or H₂O₂ generated might be involved in signal transduction of catalase, GpX, HO1 gene that can reduce oxidative stress.^{1,13,14,15} McCarthy *et.al.*⁸ reported that conjugated linoleic acid (CLA) is involved in expressing Cholesterol Scavenger receptor ABCG1, cholesterol metabolizing enzymes CYP7B1 and PGC1α target gene CPT1α this have a important role in atherosclerosis. They have further said that CLA can reduce atherosclerotic lesions, by regulating these genes that can prevent foam cell formation⁸. Our results on oxidized [1-¹⁴C] HPODE exposed to HepG2 cells also showed increased uptake by cells which indicates that it might carry out

peroxisomal degradation to H₂O₂. We have previously documented in our laboratory studies that oxidized fatty acid (HPODE and HODE) is further degraded in peroxisomes to form H₂O₂. Various genes such as Mn-SOD, catalase, nitric oxide synthase, glutathione synthesis and heme oxygenase are also been expected to be induced by HPODE and HODE effect.^{13,14,15} Darley-Usmar¹⁶ have reported that macrophages incubated with oxidized low-density lipoprotein (LDL) showed initial depletion in glutathione which was than doubled after 24h. They have stated that this response of the cells to oxidized LDL was dependent on the extent of oxidative modification of the protein. Similarly, 4-hydroxynonenal also had a similar effect on THP-1 cells. However, in absence of HepG2 cells their appears to be least degradation of [1-¹⁴C] HPODE to H₂O₂. To test 13-HPODE we subjected 13-HPODE ability to react with N-benzoyl LMB. Results have shown that there is no reduction in reactivity of peroxides with the reagent. Thus we considered the possibility that cells could generate H₂O₂ from 13-HPODE. Zhang *et al.*¹⁷ speculated that HUVECs cells treated with Ox-LDL and

H₂O₂ have showed increase in Sirt1 gene expression. In addition, to that studies by Meilhac *et al.*¹³ have reported that in rabbit femoral arterial smooth muscle cells (RFASMC), CAT protein, mRNA, and the enzyme activity were increased in response to oxidized linoleic acid (13-hydroperoxy-9,11-octadecadienoic acid [13-HPODE] and 13-hydroxy-9,11-octadecadienoic acid [13-HODE], MO-LDL, or H₂O₂. Meilhac *et al.*¹³ suggest that various cells such as endothelial cells, smooth muscle cells and macrophages are exposed to oxidative stress if they are subjected to oxidized lipids which are present in intimal area of atherosclerotic artery. In that it has been noticed that smooth muscle cells can migrate, proliferate or can show apoptotic behavior. The H₂O₂ formed can have cellular proatherogenic effects of oxidized lipids. However, studies on RFASMC showed poor uptake of 13-HPODE after prolonged exposure¹³. Furthermore, oxidized lipids and H₂O₂ can also affect various cellular processes such as cell proliferation, protein synthesis, gene expression and specific gene products. However, addition of antioxidants can show paradoxical role that is it can propagate oxidation. It has been observed that certain type of cells SV-B2 cells are found to be resistant to the effect of 13-HPODE, a peroxidized fatty acid¹². Ox-LDL in presence of HepG2 cells showed increase in the generation of H₂O₂ however, in absence of cells Ox-LDL also showed increase in lipid peroxide content at initial 1h, but was decreased at 2 and 4h. Cells possess enzymatic system that can bring about generation of H₂O₂. Our studies provide a novel approach which suggests that if peroxisomes are involved in this reaction then it shall prevent metabolic toxicity and it can also induce gene. Klammerer *et al.*¹² have reported in his studies that oxidized fats (OF) can inhibit proatherogenic effect, by reducing monocyte recruitment and SMC proliferation and migration. The OF can activate PPAR α in liver, which can lower lipid, TAG, cholesterol and VLDL levels in plasma and vasculature of rats. It has also been reported that feeding a diet rich in 13-HPODE can strongly reduce TAG levels in plasma that takes place by PPAR α -dependent pathway¹². Steinberg^{18,19} in his review have reported that monocytes/macrophages can take up Ox-LDL rapidly as compared to native LDL. There are few receptors that are involved in uptake mechanism they are scavenger receptor B (CD36), lecithin-like oxidized LDL receptor (LOX-1). The oxidized phospholipids within oxidized LDL exhibits wide variety of biological property which is supposed to be proatherogenic. Oxidized phospholipids serve as a principal epitope that is recognized by autoantibodies to Ox-LDL and scavenger receptor recognizes it as a

ligand. Oxidized cholesterol is known to have multiple effects which includes, release of MCP-1 from endothelial cells, it can be chemotactic for monocytes, can cause endothelial expression of M-CSF, enhance collagen synthesis in SMC and it can inhibit LPS-induced expression of Nf κ B, cause vasospasm by inhibiting the release/function of nitric oxide, increase VCAM gene and induce thrombosis²⁰⁻²⁶. Reports also suggest that 12/15 lipoxygenase can decrease the severity of atherosclerosis in apoE-deficient mice and LDL receptor-deficient mice. Moreover, site-specific 15-lipoxygenase expression in LDL-receptor deficient mice can accelerate atherosclerosis²⁷⁻²⁹. Kuhn and Chun have documented that 12/15 lipoxygenase may have both pro- and anti-inflammatory effects³⁰.

CONCLUSION

Thus present studies depicts the importance of enzyme lipoxygenase that converts 1-14C linoleic acid to generate 13-HPODE, 13-HODE and H₂O₂ (Scheme, Reaction 1) and 1-14C HPODE non-enzymatically forms H₂O₂ (scheme, reaction 2) and that by inducing CAT generates H₂O and O₂. In Scheme, reaction 3 oxidized LDL and lipid peroxidation forms lipid peroxides products, malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and H₂O₂ which if not treated with antioxidants such as tiron forms azelaic acid which has anti-atherosclerotic action. Azelaic acid is lipid peroxidation-derived lipophilic dicarboxylic acid. It is assumed that as in scheme reaction 4 that per oxidized lipids, 13-HPODE, 13-HODE, azelaic acid and H₂O₂ that are generated from above reactions might induce PGC1 α , PPAR α , GPx, CAT and Sirt1 gene by Nrf1 dependent manner that might prevent cellular and metabolic toxicity and increase mitochondrial biogenesis and also it might show anti-atherogenic, and anti-inflammatory property. However, studies related to Sirt1 gene expression and other transcriptional genes by above end products 13-HPODE, 13-HODE, azelaic acid H₂O₂ needs to be ascertained.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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