



Article Effect of 13-Hydroperoxyoctadecadienoic Acid (13-HPODE) Treatment on the Transcriptomic Profile of Poorly-Differentiated Caco-2 Cells

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Abstract: Dietary lipid peroxides (LOOHs) have been linked to gut pathologies including inflammatory bowel disease and cancer. As poorly differentiated (PDiff) intestinal epithelial (Caco-2) cells represent tumor cells and could model intestinal crypt cells, we investigated the cellular response of PDiff Caco-2 cells to the most common dietary LOOH, 13-hydroperoxyoctadecadienoic acid (13-HPODE), using RNA sequencing (RNA-seq). Further, we compared the results with the transcriptomic profiles of PDiff cells exposed to linoleic acid (LA) or hydrogen peroxide (H_2O_2). The results showed that 13-HPODE treatment induces expression of genes related to detoxification and several metabolic pathways including glycogen and amino acid metabolism, which may create a tumorigenic environment despite the downregulation of some proliferation-related genes. 13-HPODE also enhanced peroxisome proliferator-activated receptor signaling involved in lipid metabolism, homeostasis, and inflammation. Additionally, results indicated that 13-HPODE impacts ribosome biogenesis, phagosome, and mitochondrial function through disrupted electron transport chain, which may contribute to disease development or progression. RNA-seq results were validated using qRT-PCR. This study provides an understanding of PDiff Caco-2 cell response to 13-HPODE and the mechanisms by which 13-HPODE modulates cellular processes that may contribute to disease development or progression.

Keywords: gene expression; lipid peroxides; Caco-2 cells

1. Introduction

The human intestinal epithelium is exposed to different chemicals that we ingest with food. Among those chemicals are lipid peroxides (LOOHs) that usually form in overheated oils (fried food). LOOHs, which are by-products of pre- or auto-oxidation of polyunsaturated fatty acids (PUFAs), have been implicated in various diseases including atherosclerosis [1,2], neurodegenerative disease [3,4], rheumatoid arthritis [5], inflammatory bowel disease [6,7], and colorectal cancer (CRC) [8]. Oxidation of linoleic acid (LA), the most abundant PUFA in mammals and plants, results in the formation of 13-hydroperoxyoctadecadienoic acid (13-HPODE), which is rapidly decomposed by cells into toxic aldehydes and carboxylic acids [9,10]. Lipid peroxidation products including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) have shown to modulate gene expression and contribute to CRC [11]. In addition, frying oil has shown to worsen the inflammatory condition and tumorigenesis in the colon [12]. Caco-2 cells, which are derived



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Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). from human colorectal adenocarcinoma [13], have been widely used to study intestinal metabolism of molecules and drugs [14,15]. These cells, under standard culture conditions, have the ability to become differentiated (Diff; 14-Day) and acquire mature enterocyte-like structural and functional characteristics which makes it a suitable model to study lipid peroxidation [16–22]. On the other hand, undifferentiated or poorly differentiated (PDiff; 4-Day) Caco-2 cells mimic in vivo intestinal cancer cells and could model intestinal crypt cells, as they share the proliferative phenotype and lack of microvilli cells [23,24], which might be exposed to dietary LOOHs and contribute to disease.

It has been shown that the absorption of LA, the most common dietary PUFA, by PDiff and Diff Caco-2 cells was comparable; however, the absorption of peroxidized LA, 13-HPODE, by PDiff cells was less efficient compared to its absorption by Diff cells that possess brush borders [1]. It has been suggested that dietary oxidized fatty acids are absorbed by the intestine and are esterified into complex oxidized lipids that are transported in the plasma by lipoproteins [1]. Intestinal epithelial lipid transport by apolipoprotein B (ApoB) during differentiation depends on *microsomal triglyceride transfer protein* (MTTP), which is suppressed by nuclear receptor 2 family 1 (NR2F1) in PDiff Caco-2 cells [25]. It has been demonstrated that MTTP overexpression in PDiff Caco-2 cells increases triglyceride (TG) transfer activity and cholesterol esterification indicating the essential role of MTTP in lipid absorption [26]. Effects of linoleic acid on cells range from production of nitric oxide (NO) and reactive oxygen species (ROS) to protection against oxidative stress [27,28]. In addition, dietary PUFAs are highly susceptible to peroxidation, which has been implicated in the development of colon cancer [11]. Moreover, oral consumption of frying oil in mice has shown to aggravate colon tumorigenicity [12]. Our recent study demonstrated alterations in the expression of inflammatory genes and reduced cell viability on annexin V staining of PDiff Caco-2 cells when treated with peroxidized LA, 13-HPODE [6]. Despite these studies, the effects of dietary LOOHs on PDiff Caco-2 cells, which are sensitive to chemicals, have not been characterized fully.

PDiff Caco-2 cells are used in cancer studies because their gene expression profile resembles that of human colon cancer cells [23], and might also reflect other subpopulation cell types in the human colon [29]. Of note, intestinal proliferative crypt cells might undergo programmed cell death, under stressful conditions, to remove damaged cells and protect from tumorigenesis [30] suggesting that crypt cells could initiate gut pathology. In this study, we investigated the response of PDiff Caco-2 cells to 13-HPODE, in terms of differential gene expression, gene ontology, and pathway enrichment, using mRNA sequencing (RNA-seq) as this group of cells represents intestinal tumor cells and could also model intestinal crypt cells, which could be exposed to the stress of dietary LOOHs. In addition, studying PDiff Caco-2 cells allows us to look at a heterogeneous population of Caco-2 cells before differentiation, thus having broader plasticity in the cellular physiology and response to stress, as compared to Diff cells. We also compared our results to the transcriptomic changes of PDiff cells exposed to unoxidized LA or hydrogen peroxide (H_2O_2) , which is another source of oxidative stress and similar antioxidant response between 13-HPODE and H_2O_2 treatments was observed in smooth muscle cells [31]. We validated our RNA-seq results using quantitative RT-PCR. The overall goal of this study was to understand how the transcriptomic profile of PDiff Caco-2 cells changes in response to the most common dietary lipid peroxide, 13-HPODE. These changes in gene expression and pathway regulation could provide mechanisms that might be implicated in the development or progression of disease in intestinal crypt cells or tumor cells exposed to dietary LOOHs.

2. Materials and Methods

2.1. *Materials*

Dulbecco's modified eagle medium (DMEM), L-glutamine, penicillin/streptomycin, fetal bovine serum (FBS), phosphate-buffered saline (PBS), TRIzolTM reagent, and TURBO DNA-free kit were obtained from Invitrogen (Carlsbad, CA, USA). Trypsin-EDTA solution

was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Linoleic acid and soybean lipoxydase were purchased from Sigma (St. Louis, MO, USA). Leucomethylene blue (LMB) was purchased from Alfa Aesar (Ward Hill, MA, USA). NEBNext Ultra II Directional RNA Library Prep kit for Illumina, indexed adaptors and NEBNext Library Quant Kit were purchased from New England Biolabs (NEB; Ipswich, MA, USA). High Sensitivity D1000 reagents were obtained from Agilent (Santa Clara, CA, USA). SsoAdvancedTM Universal SYBR[®] Green Supermix and PCR Primers were purchased from BioRad (Hercules, CA, USA) and Eurofins (Luxembourg), respectively.

2.2. Cell Culture

Colorectal adenocarcinoma (Caco-2) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in DMEM and supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin, and 15% FBS. Trypsin-EDTA solution 0.25% was used to sub-culture confluent monolayers and 2.5×10^5 – 3×10^5 cells/well were seeded in 6-well plates and maintained for 4 days. All experiments were carried out on PDiff cells (Day-4 post-confluence) and run in triplicate.

2.3. Preparation of 13-Hydroperoxyoctadecadienoic Acid (13-HPODE)

13-HPODE was freshly prepared as previously described [22,32–34]. Briefly, linoleic acid (LA; 200 μ M) in PBS was oxidized with the addition of 10 units soybean lipoxydase. Conjugated diene (HPODE) formation was monitored by scanning the absorption spectrophotometrically (Uvikon XL, Biotek 125 Instruments, El Cajon, CA, USA) at optical density 234 nm; PBS was used as a reference. Lipid peroxide formation was determined using LMB assay [35]. The freshly prepared 13-HPODE was filter sterilized to minimize the risk of contamination and used immediately for the culture experiments to reduce spontaneous peroxide decomposition. No significant difference was observed in peroxide content prior and after filter sterilization of 13-HPODE, which was confirmed by LMB assay as shown in Figure S1. 13-HPODE concentration was calculated using UV spectrophotometry.

2.4. Treatment of Cells with 13-HPODE, LA or H_2O_2

PDiff Caco-2 cells were starved in serum-free medium for 3 h prior to treatments. Cells were treated with 100 μ M of either 13-HPODE, LA, or H₂O₂ for 24 h in serum-free medium. This concentration was used as it resulted in the significant differences between 13-HPODE-treated and untreated Caco-2 cells in our previous study [6]. Control (untreated) cells were maintained in serum-free medium with equal volume of PBS (500 μ L). After 24 h incubation, cells were rinsed with PBS and immediately harvested into TRIzol for total RNA isolation.

2.5. Total RNA Extraction and Quantification

TRIzol reagent was used to lyse the cells directly in the culture plates. Chloroform was added to cell lysate and samples were incubated for 3 min at room temperature then centrifuged at $12,400 \times g$ for 15 min. The aqueous phase was transferred into new tubes from which total RNA was precipitated using isopropyl alcohol and resuspended in nuclease-free water. RNA concentration, quality, and purity were tested via NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) showing absorbance ratios of > 1.8 at 260 nm and 280 nm. The TURBO DNA-free kit was used to remove co-extracted DNA from the RNA samples following the manufacturer's instructions.

2.6. RNA-seq Library Preparation and Sequencing

We used the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB, E7490S) for mRNA isolation from 500 ng of total RNA input followed by the NEBNext Ultra II Directional RNA Library Prep kit for Illumina (NEB, E7760S) for library preparation according to the manufacturer's instructions. mRNA was fragmented to a target size of 200 base pairs and ligated to indexed adaptors for Illumina sequencing (NEB, E7710, E7730). We used

NEBNext Sample Purification Beads to remove unincorporated Illumina adapters. Quality and quantity of RNA-seq libraries were assessed by using High Sensitivity D1000 reagents on the TapeStation 2200 (Agilent, Santa Clara, CA, USA), and NEBNext Library Quant Kit (NEB, E7630S), respectively. Individual libraries were combined into an equimolar sequencing pool, which was checked on the TapeStation using High Sensitivity D1000 ScreenTape assay and showed good quality. The library pool was sent for HiSeq4000 sequencing (2 \times 150 bp) to GENEWIZ (South Plainfield, NJ, USA).

2.7. Sequence Data Processing

Paired-end read quality was assessed using FastQC (version 0.11.7) [36]. Poor-quality bases were trimmed and adapters were removed using Trimmomatic (version 0.36) [37]. Retained reads were aligned to a human reference genome (Ensembl/Genome Reference Consortium Human Build 38, GRCh38) using Hisat2 (version 2.1.0) [38]. FeatureCounts (version 1.5.0) was used to count the fragments mapped to an exon/gene [39]. Differentially expressed genes (DEGs; adjusted p < 0.05) between different groups were determined using an R-package DESeq2 (version 1.30.0) [40]. One sample of the LA-treated group was excluded from the study as its differential gene expression results did not correlate with the other samples.

2.8. Gene Ontology and Pathway Enrichment Analyses

Gene Ontology (GO) analysis was performed on DEGs of adjusted p < 0.05 (from DESeq2) using clusterProfiler (version 3.18.0) R-package [41]. The function "enrichGO" of clusterProfiler was used to determine the enriched (adjusted p < 0.05) GO biological processes, molecular functions, and cellular components in treated cell groups compared to the untreated group. Pathway enrichment analysis was carried out using Generally Applicable Gene-set Enrichment (GAGE; version 2.40.0) R-package [42]. The function "gage" was used to determine the dysregulated pathways (p < 0.05) in each treatment group based on DEGs and their log2 fold change.

2.9. Validation Using qRT-PCR

Representative genes were chosen, based on differential gene expression and enrichment analysis, for validation of RNA-seq results using qRT-PCR. Eighteen genes were chosen for 13-HPODE-treated group, 12 genes for LA-treated group, and 11 genes for H₂O₂treated group. Each Ten microliters of PCR reaction consisted of 5 μ L of SsoAdvancedTM Universal SYBR[®] Green Supermix, 2.5 μ M of forward and reverse primers, and 0.1 ng of cDNA. The thermal cycling protocol included an initial denaturation step at 95 °C for 30 s followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. *GAPDH* was used as the control gene. Relative mRNA expression was calculated using the $\Delta\Delta$ Ct method, and statistical differences between treated and untreated groups were determined using the *t*-test. The data were presented as mean \pm SD. Primer sequences used in qRT-PCR are provided in Table S1.

3. Results

We treated PDiff Caco-2 cells with 13-HPODE, LA, or H_2O_2 for 24 h. Untreated cell group was maintained in PBS and used as a control. RNA extraction and processing protocol is described in the Materials and Methods section. The sequencing reads ranged between 31 and 60 million reads (Table S2) with mean quality score > 37.2. Differential gene expression, gene ontology, and pathway analysis were conducted using DESeq2, clusterProfiler and GAGE R-packages, respectively. Principal component analysis (PCA; Figure S2) and sample-to-sample distance heatmap (Figure S3) showed that samples from each group were closely similar in gene expression with respect to treatment and that untreated and treated cell groups were well separated. Genes with adjusted *p*-value < 0.05 and large mean expression across all cell groups call for significance as demonstrated in log ratio vs. mean average (MA) plots (red dots; Figure S4).

3.1. 13-HPODE-Treated PDiff Caco-2 Cells

3.1.1. Differential Gene Expression

We identified 6648 differentially expressed genes (DEGs; adjusted p < 0.05) between untreated and 13-HPODE-treated PDiff Caco-2 cells using DESeq2 (Table S3); 3345 genes were upregulated and 3303 genes were downregulated upon treating the cells with 13-HPODE. Figure 1 demonstrated a heatmap and hierarchical clustering of the top 50 DEGs in 13-HPODE-treated cell groups.



Figure 1. Differential gene expression in 13-hydroperoxyoctadecadienoic acid (13-HPODE)-treated Caco-2 cells compared to untreated cells. Heatmap shows the top differentially expressed genes (DEGs; adjusted p < 0.05) in 13-HPODE-treated (4D.H1, 4D.H2, and 4D.H3) compared to untreated (control) cells (4D.C1, 4D.C2, and 4D.C3). Green, upregulated; red, downregulated.

Treating PDiff Caco-2 cells with 13-HPODE caused upregulation of genes involved in peroxisome proliferator-activated receptor (PPAR) signaling, which has been implicated in metabolic syndrome and cancer [43]. Among those genes were *HMGCS2*, which initiates the first step of ketogenesis, *CPT1A*, which facilitates fatty acid uptake for mitochondrial beta-oxidation, *PLIN2*, which coats the surface of intracellular lipid droplets, and *FABP1*, which is involved in fatty acid uptake and transport. Genes that play a role in biosynthesis of phospholipids such as *CHKA* and *ETNK1* were upregulated indicating phospholipid metabolic alterations. Other genes involved in lipid metabolism and uptake were induced such as *A1CF*, a complementation factor of apolipoprotein B mRNA editing enzyme involved triglyceride uptake, *CREB3L3*, which is triggered by endoplasmic reticulum stress and involved in acute inflammatory response and triglyceride metabolism, and *LRP2*, which is endocytic glycoprotein receptor involved in uptake of sterols and lipoproteins.

Genes involved in amino acid metabolism such as *PHGDH* and *GPT2* were upregulated; the latter also plays a role in gluconeogenesis. These results suggest significant metabolic alterations in response to 13-HPODE treatment.

Genes involved in the regulation of cellular processes were upregulated such as CEBPA/PB, which inhibit proliferation, promote differentiation, and mediate acute phase response [44], ADM2, which regulates cardiovascular and intestinal activities and ameliorates intestinal inflammation and protects epithelial barrier [45], and ANK3, which links integral membrane proteins to cytoskeleton and regulates cellular activities. The latter's expression has shown to be increased during Caco-2 polarization [46]. In addition, TTN, which is a key component of stress fibers and brush borders [47], was upregulated. On the other hand, CCN1/N2, CYTOR, and PIM1, which promote cell proliferation, and ODC1, which is important for polyamine biosynthesis, were downregulated. Alterations of the previously mentioned genes indicate low proliferative potential and possible shift towards differentiation. Expression of DDIT4 was increased upon 13-HPODE treatment, which inhibits mTORC1 and regulates cellular processes in response to stress. Upregulated expression of AKR1C1/C2/C3 genes, which have detoxifying activity and reduce aldehydes to alcohols, may indicate conversion of 13-HPODE to aldehydes in the cells. *IDH1*, which generates NADPH, which is an important cofactor in drug metabolism, antioxidant response, and inhibition of ROS production, was also upregulated. Among downregulated genes were transporters of small molecules such as glucose transporters-SLC2A1/A3, which suggest altered transport of substances across biological membranes by 13-HPODE. Genes involved in inflammation such as CXCL1/L3, chemotactic factors, and CCL20, inflammatory chemokine, were downregulated, which may indicate an anti-inflammatory effect of 13-HPODE.

3.1.2. Gene Ontology

Biological processes that were enriched upon 13-HPODE treatment included ribosomal biogenesis, translation, RNA processing and transport, and protein targeting. In addition, DNA metabolic process, response to endoplasmic reticulum stress, and response to unfolded protein were also enriched. Intrinsic apoptotic signaling pathway and response to oxidative stress were also enriched. This suggests that a significant stress was brought to the cells by 13-HPODE. Biological processes involved in metabolism were affected as well, such as lipid catabolic process, steroid metabolic processes, regulation of carbohydrate metabolism, and cellular aldehyde metabolic process. Peroxisome organization and establishment of cell polarity were also enriched (Figure 2a; Table S4).

Among molecular functions enriched in 13-HPODE-treated cells were cadherin binding, cell adhesion molecule binding, protein serine/threonine kinase activity, coenzyme binding, and GTPase binding. In addition, ligase activity, ribonucleoprotein complex binding, transcription coactivator activity, protein kinase regulator activity, methyltransferase activity, and helicase activity were enriched (Figure 2b; Table S5).

Among enriched cellular components in 13-HPODE-treated Caco-2 cells were focal adhesion, cell–substrate junction, cell–cell junction, spindle, and cell-leading edge. Furthermore, ribosomal subunit, nuclear envelop/membrane/speck, and spliceosomal complex were enriched. In addition, outer membrane, organelle outer membrane, mitochondrial outer membrane, mitochondrial matrix, and ubiquitin ligase complex. Brush border and peroxisome were enriched as well (Figure 2c; Table S6).

3.1.3. Pathway Analysis

We found that treating PDiff Caco-2 cells with 13-HPODE led to upregulation of retinol metabolism, which could be a counter-regulatory defense mechanism against 13-HPODE, which is considered a xenobiotic. Consistent with previous reports [48], 13-HPODE induced steroid hormone biosynthesis, which has a role in maintaining intestinal barrier [49]. Additionally, starch and sucrose metabolism as well as amino acid metabolism were upregulated. Other pathways that were enriched with slightly higher p values

included PPAR signaling as well as peroxisome pathway and metabolism of xenobiotics by cytochrome P450 suggesting that 13-HPODE could trigger detoxification mechanisms. On the other hand, ribosome, RNA polymerase, spliceosome, and pyrimidine metabolic pathways were suppressed due to 13-HPODE treatment. Phagosome degradation pathway was also downregulated. Moreover, 13-HPODE treatment caused downregulation of oxidative phosphorylation indicating possible impaired mitochondrial function (Table 1).



(c)

Figure 2. Gene ontology (GO) analysis in 13-HPODE-treated poorly differentiated (PDiff) Caco-2 cells. Top enriched biological processes (a), molecular function (b), and cellular components (c) in 13-HPODE-treated cells relative to untreated (control) cells (adjusted p < 0.05).

0.01

0.02

GeneRatio

0.03

0.04

KEGG Pathway (Upregulated)	p Value
hsa00830 Retinol metabolism	0.002
hsa00140 Steroid hormone biosynthesis	0.005
hsa00500 Starch and sucrose metabolism	0.038
hsa00260 Glycine, serine and threonine metabolism	0.059
hsa03320 PPAR signaling pathway	0.059
hsa04146 Peroxisome	0.063
hsa00980 Metabolism of xenobiotics by cytochrome P450	0.068
KEGG Pathway (Downregulated)	p Value
hsa03010 Ribosome	0.0002
hsa03008 Ribosome biogenesis in eukaryotes	0.0008
hsa03040 Spliceosome	0.001
hsa00240 Pyrimidine metabolism	0.004
hsa04145 Phagosome	0.016
hsa03020 RNA polymerase	0.024
hsa03060 Protein export	0.032
hsa00190 Oxidative phosphorylation	0.036

Table 1. Pathway enrichment analysis in 13-HPODE-treated PDiff Caco-2 cells. Pathway analysis demonstrates upregulated and downregulated Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in 13-HPODE-treated cells relative to untreated (control) cells.

3.2. LA-Treated PDiff Caco-2 Cells

3.2.1. Differential Gene Expression

We identified 4225 differentially expressed genes (adjusted p < 0.05) between untreated and LA-treated PDiff Caco-2 cells. Of these, 2080 genes were upregulated and 2145 genes were downregulated in LA-treated cells compared to untreated cells (Table S7). As in 13-HPODE-treated cells, genes involved in lipid uptake and metabolism such as FABP1 and HMGCS2 were also induced upon LA treatment. APOL6, which has a role in lipid transport in the cytosol, was also upregulated. On the contrary, other genes of lipid metabolism were downregulated including MGLL, which hydrolyzes monoacylglycerol to fatty acid and glycerol, and OLR1, which promotes internalization of oxidized LDL. PCK2 involved in glucose production was upregulated as well. DDIT4 and CHAC1, which have a role in stress and unfolded protein response, were upregulated. CEBPB, which have shown to inhibit proliferation, promote differentiation, and mediate acute phase response [44], and IHH, that regulates proliferation and promotes differentiation [50], were also upregulated. On the other hand, similar to 13-HPODE-treated cells, RGCC, CCN1/N2, PIM1, and CYTOR were downregulated in LA-treated cells, which may indicate reduced proliferative potential and enhanced differentiation of PDiff cells by both treatments. While some small molecule transporters such as SLC7A11, an anionic amino acid transporter, were upregulated; others were downregulated such as SLC20A1, a sodium-phosphate symporter, suggesting altered membrane transport of substrates by LA. Reduced expression of CXCL1 and CCL20 chemotactic factors was also seen indicating anti-inflammatory effect of LA. The top 50 DEGs upon LA treatment are shown in Figure 3.

3.2.2. Gene Ontology

Among enriched biological processes upon treating PDiff Caco-2 cells with LA were coenzyme metabolic process, regulation of catabolic process, response to insulin, and autophagy. Nucleobase-containing compound transport, ribonucleotide, and nucleotide biosynthetic processes were also enriched. Similar to 13-HPODE-treated cells, we observed enrichment of response to endoplasmic reticulum stress and intrinsic apoptotic signaling pathway in LA-treated cells. Furthermore, alcohol, lipid, and glucose metabolic processes were enriched indicating significant impact of LA on cellular metabolism (Figure 4a; Table S8).





LA caused enrichment of molecular functions such as cadherin binding, cell adhesion molecule binding, ligase activity, and GTPase binding, which were also enriched upon 13-HPODE treatment. In addition, phosphatase activity, tRNA binding, and phospholipid binding were enriched in LA-treated cells (Figure 4b; Table S9).

As in 13-HPODE-treated cells, cellular, organelle, and mitochondrial outer membranes, nuclear envelop/speck/pore, focal adhesion, cell–substrate junction, cell leading edge, and spindle were among the enriched cellular components in LA-treated cells. Endoplasmic reticulum lumen, vacuolar membrane—that stores nutrients or waste products—and lysosomal membrane were enriched as well as the peroxisomal part (Figure 4c; Table S10).

3.2.3. Pathway Analysis

Pathway analysis revealed that most significantly enriched pathways in LA-treated cells were downregulated. Although ribosome biogenesis was downregulated, the aminoacyl-tRNA biosynthesis pathway, which is essential for protein synthesis, was marginally upregulated in LA-treated cells. The starch and sucrose metabolism and peroxisome pathway were also upregulated, with slightly higher *p* values, upon treating cells with LA. On the other hand, phagosome and lysosome pathways were downregulated. In addition, spliceosome and pyrimidine metabolism were downregulated. NOD-like receptor signaling involved in immune response was also suppressed (Table 2).







(b)



(c)

Figure 4. Gene Ontology (GO) analysis in LA-treated PDiff Caco-2 cells. Top enriched biological processes (**a**) molecular functions (**b**) and cellular components (**c**) in LA-treated cells relative to untreated (control) cells (adjusted p < 0.05).

KEGG Pathway (Upregulated)	p Value
hsa00970 Aminoacyl-tRNA biosynthesis	0.079
hsa00500 Starch and sucrose metabolism	0.111
hsa04146 Peroxisome	0.163
KEGG Pathway (Downregulated)	p Value
hsa04145 Phagosome	0.005
hsa03008 Ribosome biogenesis in eukaryotes	0.012
hsa03040 Spliceosome	0.013
hsa04621 NOD-like receptor signaling pathway	0.015
hsa00240 Pyrimidine metabolism	0.018
hsa04142 Lysosome	0.021

Table 2. Pathway enrichment analysis in LA-treated PDiff Caco-2 cells. Pathway analysis demonstrates upregulated and downregulated KEGG pathways in LA-treated cells relative to untreated (control) cells.

3.3. H₂O₂-Treated PDiff Caco-2 Cells

3.3.1. Differential Gene Expression

We identified 5639 DEGs (adjusted p < 0.05) between untreated and H₂O₂-treated PDiff Caco-2 cells. Of these, 2743 genes were upregulated and 2896 were downregulated upon treatment of cells with H₂O₂ (Table S11). Similar to 13-HPODE-treated cells, we observed downregulation of PIM1, CCN1/N2, and CYTOR in H₂O₂-treated cells suggesting reduced cellular proliferation. SLC38A2, an amino acid transporter, SLC20A1, a phosphate transporter, and EPHA2 (ECK), which has a role in intestinal homeostasis and barrier function [51], were suppressed which might suggest impairment of molecule transport and intestinal barrier upon treating the cells with H_2O_2 . In addition, *DKK1*, a Wnt signaling inhibitor, DUSP6/P4, which dephosphorylate and inactivate MAP/ERK kinase, FAT1, which has a role in cell polarization, and JAG1, which is involved in notch signaling, were downregulated, which may also suggest disrupted intestinal homeostasis. Tumor suppressor genes such as *KLF6* and *MAFF* which also has a role in stress response [52], were downregulated. H_2O_2 may affect lipid metabolism as it induced the expression of HMGCS2, A1CF and HSDL2, which may have a role in fatty acid metabolism [53], while it suppressed other genes such as PTGS2 and OLR1. H₂O₂ treatment caused upregulation of antioxidant genes such as BPNT1, which has a detoxifying effect by preventing accumulation of phosphoadenosine phosphate (PAP) [54], CAT, which has a protective effect against H_2O_2 , and IDH1, which is involved in NADPH production and reduces the production of ROS [55]. The top 50 DEGs upon treating PDiff cells with H_2O_2 are shown in Figure 5.

3.3.2. Gene Ontology

 H_2O_2 treatment caused enrichment of mRNA catabolic process and rRNA processing, ribosome biogenesis, and translation initiation as well as processes involved in protein localization and targeting to membrane and ER, which were also enriched by 13-HPODE treatment. Consistent with previous studies [56–58], H_2O_2 led to enrichment of doublestrand break repair. Similar to 13-HPODE, we also observed enrichment of apoptotic signaling pathway and response to oxidative stress due to H_2O_2 treatment. Among other enriched biological processes were autophagy, carboxylic acid catabolic process, and cholesterol biosynthetic process (Figure 6a; Table S12).



Figure 5. Differential gene expression in H_2O_2 -treated Caco-2 cells compared to untreated cells. Heatmap shows the top differentially expressed genes (DEGs; adjusted p < 0.05) in H_2O_2 -treated (4D.h2o2.1, 4D.h2o2.2, and 4D.h2o2.3) compared to untreated (control) cells (4D.C1, 4D.C2, and 4D.C3). Green, upregulated; red, downregulated.

Among enriched molecular functions upon H_2O_2 treatment were cadherin binding, cell adhesion molecule binding, and GTPase binding, which was also seen in 13-HPODE treatment. Ribonucleoprotein complex binding, RNA binding, nuclear hormone receptor binding, and ubiquitin protein ligase binding were also enriched upon H_2O_2 treatment. Enriched activities included helicase, transcription coactivator, translation factor, protein serine/threonine kinase, and protein tyrosine phosphatase. In addition, coenzyme binding and retinoic acid receptor binding were enriched by H_2O_2 (Figure 6b; Table S13).

There was a relative similarity in the enriched cellular components between 13-HPODE and H_2O_2 . Among enriched cellular components upon H_2O_2 treatment were focal adhesion, cell–cell junction, cell leading edge, cell cortex, and ruffle. Components involved in cell division such as midbody and spindle, nuclear components such as nuclear envelope, speck, and transcription factor complex were also enriched. Other enriched components included vacuolar membrane, lysosomal membrane, and mitochondrial matrix. H_2O_2 also caused enrichment of peroxisome, chromosome, and kinetochore (Figure 6c; Table S14).



(a)







Figure 6. Gene Ontology (GO) analysis in H_2O_2 -treated PDiff Caco-2 cells. Top enriched biological processes (**a**) molecular functions (**b**) and cellular components (**c**) in H_2O_2 -treated cells relative to untreated (control) cells (adjusted p < 0.05).

3.3.3. Pathway Analysis

 H_2O_2 treatment of PDiff Caco-2 cells led to upregulation of steroid and steroid hormone biosynthesis. It also promoted detoxifying pathways including peroxisome and cytochrome P450, which were also upregulated in 13-HPODE-treated cells, as well as glutathione metabolism. Consistent with a previous report that showed increased lipid uptake by ROS [59], fat digestion and absorption as well as propanoate metabolic pathways appeared to be upregulated by H_2O_2 . Metabolism of amino acids was also affected by H_2O_2 .

Similar to what we observed in 13-HPODE-treated cells, pathways involved in RNA processing and translation such as ribosome, RNA transport, and spliceosome were down-regulated in upon treatment with H_2O_2 . Pathways involved in cell adhesion were also downregulated including cell adhesion molecules (CAMs), adherens junction, focal adhesion, and tight junction suggesting significant disruption of epithelial barrier. In addition, Wnt signaling pathway was suppressed, which affects the regulation of physiological processes such as proliferation and differentiation, and immune response (Table 3).

Table 3. Pathway enrichment analysis in H_2O_2 -treated PDiff Caco-2 cells. Pathway analysis demonstrates upregulated and downregulated KEGG pathways in H_2O_2 -treated cells relative to untreated (control) cells.

KEGG Pathway (Upregulated)	p Value
hsa00280 Valine, leucine and isoleucine degradation	0.001
hsa04975 Fat digestion and absorption	0.015
hsa00140 Steroid hormone biosynthesis	0.016
hsa04146 Peroxisome	0.020
hsa00982 Drug metabolism-cytochrome P450	0.022
hsa00100 Steroid biosynthesis	0.031
hsa00260 Glycine, serine and threonine metabolism	0.043
hsa00640 Propanoate metabolism	0.049
hsa00480 Glutathione metabolism	0.062
KEGG Pathway (Downregulated)	p Value
hsa03010 Ribosome	0.0001
hsa03008 Ribosome biogenesis in eukaryotes	0.002
hsa04145 Phagosome	0.004
hsa03013 RNA transport	0.011
hsa03040 Spliceosome	0.018
hsa00240 Pyrimidine metabolism	0.020
hsa04514 Cell adhesion molecules (CAMs)	0.029
hsa04670 Leukocyte transendothelial migration	0.031
hsa04520 Adherens junction	0.034
hsa04512 ECM-receptor interaction	0.036
hsa04510 Focal adhesion	0.045
hsa04530 Tight junction	0.045

3.4. Validation of RNA-seq Results

To validate RNA-seq results using qRT-PCR, we selected three different sets of representative genes based on differential gene expression and pathway enrichment results in each treatment. We investigated 18 genes in 13-HPODE-treated cells, 12 genes in LA-treated cells, and 11 genes in H_2O_2 -treated PDiff Caco-2 cells.

Genes of PPAR signaling that regulate lipid and glucose metabolism including *PLIN2*, *HMGCS2*, *CPT1A*, *FABP1*, and *PCK1* were upregulated in 13-HPODE-treated cells (Figure S5a–e). We also tested key genes involved in stress response, detoxification, and peroxisome such as *CREB3L3*, *DDIT4*, *RDH10*, *AKR1C3*, *PEX6*, *CAT*, and *CROT*, which were upregulated in 13-HPODE-treated cells (Figure S5f–l). We observed upregulation of the brush border component *TTN*, and downregulation of *RGCC* and *ODC1* indicating reduced proliferation and shift toward differentiation (Figure S5m–o). Among downregulated genes in

13-HPODE-treated cells were *COX20, STX7*, and *CXCL1* involved in oxidative phosphorylation, phagosome and inflammation, respectively (Figure S5p–r).

In LA-treated PDiff cells, we tested genes of lipid metabolism *FABP1* and *HMGCS2*, glycogen branching enzyme *GBE1*, peroxisome-related genes *PEX6* and *CROT*, and stress response genes *DDIT4* and *CHAC1*, which showed increased expression due to LA treatment (Figure S6a–g). Upregulation of *CEBPB* and downregulation of *RGCC* and *DUSP4* was observed indicating altered proliferative phenotype and physiological processes (Figure S6h–j). Inflammatory genes *IL18* and *CXCL1* were also downregulated upon LA treatment suggesting anti-inflammatory effect of LA on PDiff cells (Figure S6k,I).

In H₂O₂-treated PDiff cells, we tested genes of detoxification including peroxisome genes *CROT* and *CAT*, glutathione transferase *GSTA4*, and aldo/keto reductase *AKR1C3*, which were upregulated upon H₂O₂ treatment (Figure S7a–d). In addition, *DDIT4*, involved in DNA damage stress response, was upregulated (Figure S7e). Genes of cholesterol synthesis and lipid metabolism were also upregulated due to H₂O₂ treatment including *MVK*, *HMGCS2*, and *FABP1* (Figure S7f–h). Among downregulated genes were *STX7*, *CXCL1*, and *DKK1* involved in phagosome, inflammation, and intestinal homeostasis, respectively (Figure S7i–k). The results of qRT-PCR were consistent with RNA-seq data.

4. Discussion

Linoleic acid, the most common dietary PUFA, can be ingested in the peroxidized form when vegetable oil is overheated such as in fried food. Studies have reported both harmful and possibly beneficial effects of LOOHs. DNA damage has been observed upon treating Caco-2 cells with subcytotoxic levels of LOOHs [60]. Peroxidized LA has also shown to exert pro-inflammatory effects and cause cell death in both Diff and PDiff Caco-2 cells [6]. Moreover, LOOHs have been strongly linked to atherogenesis [61,62], inflammatory disease [63], aging [64], and cancer [65]. On the other hand, a protective effect of LOOHs that promotes apolipoprotein A1 (ApoA1) production, in Diff and PDiff Caco-2 cells, which reduces atherosclerosis has been reported [34,66]. Of note, oxidized metabolites of LA are known to be ligands of PPARs, which are important for intestinal cell differentiation and play a role in lipid metabolism [67]. In this study, we demonstrate, using RNA-seq, that treating PDiff Caco-2 cells with peroxidized linoleic acid, 13-HPODE, can alter their transcriptomic profile significantly, which may reflect the effects of dietary LOOHs on in vivo intestinal crypt or tumor cells. We also compare the effects of 13-HPODE on PDiff cells with the effects of unoxidized LA as well as H_2O_2 , which is another source of oxidative stress.

We demonstrate that 13-HPODE treatment of PDiff Caco-2 cells caused upregulation of mechanisms that help cells in detoxification as PDiff cells are more sensitive to xenobiotics than Diff cells [29,32,68]. Among the upregulated pathways were retinol metabolism, peroxisome, and cytochrome P450. Upregulation of these pathway suggests that there is a need for induction of antioxidant defense response against 13-HPODE-derived oxidative stress to prevent cellular damage. Upregulated retinol metabolism might be a protective mechanism as retinol is considered as a non-enzymatic antioxidant [69]. Products of peroxidized lipids have significantly shown to affect retinoid metabolism [70]. We observed upregulation of beta-carotene oxygenase, *BCO1*, and retinol dehydrogenase, *RDH10*, in 13-HPODE-treated cells. The expression levels of these two genes have shown to be low in cirrhotic rat intestine, which indicates retinol's link to disrupted intestinal epithelial homeostasis [71]. In addition to its role in triglyceride and cholesterol metabolism [72], retinol is important for cellular growth, proliferation, and differentiation of intestinal epithelium and presents in higher amounts in intestinal crypts than villus cells [73]; therefore, enhancement of retinol could be a benefit of 13-HPODE.

PDiff cells showed upregulation of peroxisomal pathway in 13-HPODE-treated cells, which metabolizes lipids, detoxifies radicals, and maintains redox homeostasis. *PEX6* gene involved in peroxisome biosynthesis and protein import to peroxisomes was upregulated as well as the detoxifying *CAT* in 13-HPODE-treated cells. Increased peroxisome proliferation

and activity has been reported when PPAR ligands such as fatty acids were used [74]. CROT, IDH1, and ABCD1 genes were also upregulated enhancing peroxisomal lipid metabolism, a mechanism that some cancer cell types use to maintain tumorigenicity and survival through fatty acid oxidation [75]. We observed marginal induction of peroxisomal pathway as well as upregulation of PEX6, ACOX2, and CROT genes in LA-treated cells. This supports studies reported induction of PPAR by LA [76] and induction of peroxisomal acyl-CoA oxidase, which is associated with increase peroxisome number, in LA-treated cultured rat hepatocytes [77]. We also showed that 13-HPODE upregulated the metabolism by cytochrome P450 in PDiff cells, which allows the cells to oxidize and metabolize lipids, and clear xenobiotic compounds including dietary LOOHs and their metabolites such as 4-HNE [78]. Among upregulated genes were UGT2B4, CYP2B6, MAOB, and SULT2A1. These results suggest that exposure of intestinal epithelial cells to 13-HPODE may promote pre-systemic metabolism and reduced bioavailability of xenobiotics as they pass through the intestinal epithelium. Although peroxisomes and cytochrome P450 have a role in detoxifying substrates and protecting cells from oxidative stress, they can also produce ROS and lipid peroxidation, which might form adducts creating an environment for development of carcinogenesis and disease progression [74,79]. In addition, peroxisomal proliferation has been linked to tumor development, and induction of intestinal cytochrome P450 has been linked to gastrointestinal cancer [80–82].

Treatment of PDiff Caco-2 cells with 13-HPODE caused upregulation of *STS*, *CYP17A1*, and *AKR1C2/C3* genes involved in steroid hormone biosynthesis, which has been shown to affect intestinal function and transport of nutrients [83]. Aldo/keto reductases, AKR1Cs, also play a role in bile acid binding and detoxification by reducing lipid peroxide product, 4-HNE, to battle oxidative stress-related injury [84]. Thus, upregulated expression of AKR1Cs might be an indicative of increase intracellular toxic aldehydes. In addition, *AKR1C3* has been studied as a marker for progression of colorectal and prostate cancers in which aldehyde levels appear to be elevated [8,85,86]. Therefore, AKR1Cs may provide a potential therapeutic strategy to treat aldehyde-related pathologies. Of note, increased expression of AKR1Cs has been implicated in the resistance of anti-colon cancer therapies [87], which proposes a mechanism by which dietary LOOHs could promote drug resistance through upregulation of the detoxifying AKR1Cs.

ROS have been strongly linked to the initiation and progression of colorectal cancer (CRC) [88]. In addition, not only LOOHs have been implicated in the pathogenesis of CRC [89] but also levels of lipid peroxidation products due to oxidative stress have shown to be elevated in CRC tissue compared to normal colon mucosa [90]. Moreover, increased antioxidant enzyme activity in CRC has been reported which enhances adaptive response that enables cells to battle stress [90]. This is consistent with our results showing increased expression of genes involved in antioxidant defense mechanisms. Within this context, we observed upregulation of several Nrf2-regulated genes involved in antioxidant defense mechanisms in 13-HPODE-treated cells. Among those were genes involved in glutathione (GSH) antioxidant system such as SLC7A11, which facilitates cystine essential for GSH synthesis, GCLC, the rate-limiting step of GSH synthesis, GSR, which maintains high levels of reduced GSH, and GSTA2, that detoxifies compounds by conjugating them to GSH. We also observed upregulation of quinone reductase, NQO1, and CAT, which also have a role in detoxification of ROS and xenobiotics. This is consistent with previous studies demonstrated activation of Nrf2-mediated antioxidant response via oxidized lipids [91,92]. Moreover, cytochrome p450 oxidoreductase, POR, which catalyzes formation of tocopheryl hydroquinone, which is a powerful antioxidant, was upregulated in 13-HPODE-treated cells. This antioxidant response could be triggered by 13-HPODE exposure, disturbed redox status, oxidative stress, or a combination of them. In LA-treated cells, there was upregulation of some Nrf2-targeted antioxidant genes including SLC7A11, GCLC, GSR, and CAT; however, 13-HPODE triggered a more powerful antioxidant response than LA. It is worth to mention that none of the lipoxygenase genes were differentially expressed in either 13-HPODE- or LA-treated cells. However, PTGS2 (also called COX2), which

has been shown to be overexpressed in some cancers including CRC [93], appeared to be downregulated in both 13-HPODE- and LA-treated cells. This is consistent with a previous study reported that 13-HPODE inhibited prostaglandin synthesis by inhibiting cyclooxygenase activity [94]. Additionally, several unsaturated FAs including LA have been previously reported as COX-2 inhibitors [95]. Our results implicate that dietary peroxidized LA may promote a strong antioxidant response in the proliferative intestinal tumor or crypt cells to battle the stress derived from the exposure to LOOHs. Of note, the ability of PDiff Caco-2 cells, which are considered cancerous, to induce antioxidant response upon 13-HPODE treatment might lead to progression of disease as it enables cancer cells to survive under stress, which has been implicated in anti-cancer drug resistance [96]. In normal crypt cells, antioxidant response might help in preventing cellular damage that can be caused by oxidative stress and lead to disease. However, Wistar rats treated with repeatedly heated cooking oil, which contains high levels of LOOHs, have shown abnormal colonic epithelium (polyp formation) with aberrant crypts despite the increased antioxidant enzyme activity [97]. Taken together, these observations warrant further studies to determine the role of LOOH-induced antioxidant response in protecting cells or induction of mutagenic changes.

We showed that 13-HPODE enhanced PPAR signaling in PDiff cells consisting with the studies reported oxidized LA as a PPAR ligand [34,67,98]. This might suggest the ability of PDiff cells to uptake 13-HPODE and induce PPAR signaling despite the lack of brush borders. Induction of PPAR signaling leads to activation of downstream genes involved in glucose and lipid metabolism including beta-oxidation. We observed upregulation of PPARA gene and some of its downstream genes including HMGCS2, FABP1, and CPT1A genes involved in ketogenesis, uptake of FA, and mitochondrial beta-oxidation, respectively, as well as *PLIN2* that coats lipid droplets. We also observed upregulation of *FGF21*, which is required for activation of FA oxidation and ketogenic pathways and expression of PPARA target genes [99]. PCK1 and AQP7 were also upregulated suggesting that gluconeogenesis might be enhanced by 13-HPODE. Activation of PPAR could also be a protective antiinflammatory response against 13-HPODE [100] as we also observed downregulation of proinflammatory genes including IL18, CCL20, and CXCL1/L3/L8. Previous studies have shown reduced expression of chemokines CXCL1/L8 when expression level of PPAR α was increased indicating anti-inflammatory effect [101,102]. Another study has shown reduced expression of *IL18* in mice treated with PPAR α agonist, Wy14643 [103]. Moreover, it has been reported that fenofibrate, another PPAR α agonist, also reduced expression of chemokines, including CCL20, in HT-29 cells [104]. On the other hand, previous reports suggested that PPAR activation in the intestinal stem cells may have pro-tumorigenic effect, which indicates that exposure of intestinal crypt cells or tumor cells to 13-HPODE might contribute to the development or progression of cancer [105]. It is worth to note that 13-HPODE caused upregulation of SRC proto-oncogene, which has shown to be upregulated in CRC [106]. Additionally, increased expression of PLIN2 might indicate increased lipid droplets, which have shown to play a role in colon cancer progression [107]. Other reports suggested that PPAR signaling induces cell differentiation or apoptosis [108] and reduces proliferation through downregulation of MYC proto-oncogene [109,110], which we observed, in our study, in 13-HPODE-treated cells. These conflicting studies and our results might indicate dual effects of 13-HPODE-derived PPAR signaling on PDiff cells although the role of PPARs in tumor development requires further investigations.

As seen in this study, starch and sucrose metabolic pathway was enhanced by 13-HPODE treatment of PDiff Caco-2 cells. *GYS2* and *GBE1* genes, involved in glycogen synthesis and solubility, *AGL* and *PYGM*, involved in glycogen degradation, appeared to be upregulated suggesting alteration of cellular glycogen contents. In addition, glycogen metabolism has shown to play an essential role in cancer cell survival [111]. We also observed upregulation of amino acid metabolism in 13-HPODE-treated cells, in particular glycine, serine, and threonine metabolism, which also plays a role in sustaining cancer cell survival. We also observed upregulation of *PHGDH* involved in serine biosynthesis, which has also shown to maintain survival [112]. The significant impact of 13-HPODE on glycogen and amino acid metabolism of PDiff cells proposes a mechanism by which dietary LOOH may alter the metabolic processes of intestinal crypt or tumor cells to create an environment that initiates or promotes gut disease. Of note, intestinal crypt cells have the ability to initiate tumors especially if exposed to oxidative stress [113].

Components involved in physiological processes such as cell growth, differentiation, and motility were enriched in all three treatment groups. Among those were focal adhesion, cell–substrate junction, cell–cell junction, and cell leading edge. Previous reports indicated that oxidative stress including oxidized lipids could affect cellular barrier function through alteration of focal adhesion, adherens junction, and cell cytoskeleton [114,115]. We also observed downregulation of *CLDN1*, which is a barrier-forming tight junction gene and upregulation of the pore-forming *CLDN2* in 13-HPODE-treated cells, a characteristic of IBD [116]. In addition, it has been reported that LA and its oxidized metabolite could modulate cellular differentiation of Caco-2 cells [117–119].

PDiff Caco-2 cells showed downregulation of pathways involved in RNA processing and translation including ribosome and spliceosome as well as pyrimidine metabolism in the three treatment groups. This alteration might indicate reduced proliferation as these pathways have shown to be downregulated when Caco-2 cells lose their proliferative potential and shift towards differentiation [120]. In addition, induction of intestinal cell differentiation by 13-HPODE or LA has been previously reported [34,121]. Alternatively, downregulation of the translation machinery could be due to oxidative damage to the ribosomes to which the cells needed a feedback inhibition of ribosome biogenesis to prevent translation errors and further ribosomal damage [122]. Of note, oxidative stress-induced modification of RNA has been linked to age-related diseases including neurodegenerative disorders [122]. Moreover, we observed increased expression of pro-apoptotic genes such as DDIT4, BMF, BNIP3L, and BCL2L14 and decreased expression of anti-apoptotic BCL2L1 in both 13-HPODE- and LA-treated groups suggesting reduced cell viability. This is consistent with previous studies reported that 13-HPODE increases cell death [6] and that LA induces apoptosis and cell differentiation [121] in Caco-2 cells. In addition, our results at the gene expression level showed downregulation of several genes involved in proliferation such as CDK10, PIM1, CCN1/N2, and CYTOR in both 13-HPODE- and LA-treated cells, which suggests reduced proliferation. Although our results suggest, in part, that 13-HPODE promotes cell death, they may also indicate that 13-HPODE could trigger antioxidant response and modulate metabolic processes in a way that creates a carcinogenic environment and renders cells fight stress which could explain the resistance of some cancer types to anticancer therapies.

13-HPODE treatment of PDiff Caco-2 cells caused downregulation of oxidative phosphorylation. We observed downregulation of genes involved in electron transport chain complexes including complex I, III, IV, and V in 13-HPODE-treated cells. Our results support studies that demonstrated that oxidized LA products can damage the mitochondria and interfere with oxidative phosphorylation as these products can incorporate into mitochondrial membrane lipids and produce more radicals [123,124]. In addition, PUFAs have shown to increase mitochondrial β -oxidation and ROS production, which might cause mitochondrial dysfunction [124]. Of note, lipid peroxidation-related mitochondrial dysfunction has been linked to several pathologies including non-alcoholic steatohepatitis, ischemic cardiac reperfusion, and IBD [125–127].

We observed downregulation of the cellular phagosomal pathway in PDiff Caco-2 cells for all treatment groups. Among downregulated genes were those that encode subunits of vacuolar-ATPase involved in organelle acidification, such as *ATP6V1D/V0B*. Intestinal epithelial cells are considered non-professional phagocytic cells and this effect of reduced phagosomal pathway could be attributed to the reduction of oxidative phosphorylation, particularly in 13-HPODE-treated cells, as phagocytosis is known to be an energy-dependent process [128,129]. It has been demonstrated that modification of photoreceptor outer segments by lipid peroxidation products causes impairment of lysosomal degradation [130], suggesting the negative regulation of lysosomal/phagosomal degradation by LOOH adducts. Of note, impaired intestinal epithelial autophagy has been implicated in the pathology of IBD [131], and impaired intestinal epithelial phagosomal function may affect the ability of epithelial cells, the first line of defense in the gut, to clear the pathogens they encounter [128]. Thus, disruption of both oxidative phosphorylation and phagosomal pathway could propose a mechanism by which dietary LOOHs contribute to IBD and/or IBD-related carcinogenesis in intestinal crypt or tumor cells.

On the other hand, PDiff Caco-2 cells showed an anti-inflammatory response to either treatment evidenced by downregulation of inflammatory chemokines, *CXCL1/L3* and *CCL20*, and NOD-like receptor signaling. We demonstrated that upregulation of PPAR signaling by 13-HPODE might exert an anti-inflammatory response, which is consistent with a previous study reported the protective anti-inflammatory effect of PPAR against lipopolysaccharide [132]. Furthermore, PPAR ligands have shown to reduce expression of TNF α as well as chemokine receptors and chemokines [133] including *CXCL1* [134]. Previous studies also reported anti-inflammatory properties of PUFAs [135–137] consisting with our results.

 H_2O_2 is a source of oxidative stress that can be found in food preservatives, dental care products, and in some beverages consumed daily such as coffee and tea, and might predispose the intestinal epithelium to oxidative stress [138]. To evaluate whether H_2O_2 has a similar impact of 13-HPODE on the transcriptomic profile of Caco-2 cells, we treated PDiff cells with 100 μ M H₂O₂. A previous study reported that PDiff Caco-2 cells maintained 95% viability when treated with 100 μ M of H₂O₂ and that cellular and DNA damage was observed at 150 μ M H₂O₂ or higher [58]. H₂O₂ is normally detoxified by peroxisomal catalase and glutathione defense mechanism [115]. Similar to what we observed in 13-HPODE-treated cells, H₂O₂-treated cells showed upregulation of multiple pathways involved in detoxification such as peroxisome, cytochrome P450, and glutathione metabolism suggesting disrupted redox status in both treatment groups. We observed upregulation of CAT, PEX6, and PEX11A, as well as peroxisomal lipid metabolism genes including *IDH1*, *ABCD1*, and *CROT* in H_2O_2 -treated cells. It has been reported that H_2O_2 may cause peroxisomal proliferation supporting the antioxidant role of peroxisome against ROS [74]. Upregulated genes also included alcohol dehydrogenases ADH5/6, and UGT2B4 involved in metabolism of toxic compounds and xenobiotics. Nrf2-mediated antioxidant response was also promoted by H_2O_2 evidenced by upregulation of MGST3 and GSTA4/A2/M4 involved in glutathione metabolism indicating disrupted redox status and promoted defense against oxidative stress. On the other hand, cellular cytoskeleton and integrity were disturbed by H_2O_2 as cell adhesion molecules, adherens/tight junction, and focal adhesion pathways were downregulated. This is consistent with previous studies reported Caco-2 cells' injury and barrier dysfunction in response to oxidative stress, which contributes to IBD [139,140]. In addition, intestinal crypt cells have the ability to initiate tumors if exposed to oxidative stress [113].

We demonstrated that H_2O_2 treatment upregulated lipid uptake and metabolism as we observed upregulation of *FABP1*, *MTTP*, and *DGAT2*. We also observed that H_2O_2 induced steroid hormone and steroid biosynthesis, which has been observed in studies of liver cells treated with H_2O_2 [59,141]. *LSS*, *DHCR24*, and *MSMO1* involved in steroid biosynthesis were upregulated in H_2O_2 -treated cells. Upregulation of steroid biosynthesis might suggest sustained tumorigenicity as cholesterol synthesis is considered a hallmark feature of cancer [142]. Steroid biosynthesis was not affected by 13-HPODE treatment; in fact, it has been previously reported that oxidized lipids could inhibit cholesterol synthesis by binding to SREBPs and suppressing their nuclear localization [143]. We observed that H_2O_2 caused alteration of amino acids metabolism including upregulation of valine, leucine and isoleucine degradation pathway, which have also shown to be upregulated in MCF-7 cells treated with H_2O_2 ; this pathway has been linked to volatile organic compounds, which are biomarkers for cancer [144]. Our findings suggest that H_2O_2 might have a significant impact on intestinal tumor or crypt cells, which alters redox status and modifies lipid and amino acid metabolism as well as cell cytoskeleton affecting cellular physiology, integrity, and survival fates.

Table 4 demonstrates comparative differential expression of selected genes between the three treatment groups. Collectively, our results indicated that 13-HPODE, LA and H_2O_2 upregulated the. peroxisomal pathway and peroxisome-related genes including *PEX6* and *CROT*. Although we observed upregulation of genes involved in PPAR signaling in both 13-HPODE- and LA-treated cells including PLIN2 and HMGCS2, the upregulation effect was more pronounced in 13-HPODE-treated cells. This may suggest that 13-HPODE is a more potent activator of PPAR signaling than LA although both oxidized and unoxidized LA have previously shown to activate PPARs [34,76]. Moreover, gene expression and pathway analysis indicated that 13-HPODE and H_2O_2 promoted a powerful antioxidant response in PDiff cells compared to LA treatment. This was evidenced by upregulation of genes involved in cytochrome P450, Nrf2-mediated response, and glutathione metabolism, including CAT and GSTA2, which might promote adaptive response. In addition, amino acid metabolism upregulation observed in 13-HPODE- and H₂O₂-treated cells, but not in LA-treated cells, might suggest that these two treatments could promote a tumorigenic environment as amino acid metabolism including serine biosynthesis has a role in cell survival [112]. 13-HPODE caused significant downregulation of oxidative phosphorylation, which was not seen in LA or H_2O_2 treatments indicating the damaging effect might be caused by 13-HPODE on mitochondrial function as reported by previous studies [123,124]. As we mentioned earlier, all three treatments caused downregulation of ribosome biogenesis, spliceosome, and pyrimidine synthesis. Downregulation of these pathways and some proliferation-related genes such as ODC1 and RGCC might indicate reduced proliferative potential as well as possible oxidative damage to ribosomes to which the cells responded by downregulating ribosome biogenesis in order to prevent translation errors. Despite the downregulation of some inflammatory genes including CXCL1 and IL18 in all three treatments, the phagosomal pathway also appeared to be downregulated, which has been implicated in the pathology of bowel disease [131]. Gene ontology analysis showed enrichment of focal adhesion and cellular junctions in all three treatments, however, pathway analysis showed significant downregulation of cell adhesion molecules, adherens junction, and focal adhesion in H_2O_2 -treated cells indicating damaging effect of H_2O_2 on cellular cytoskeleton and junctions, which might affect barrier function [139,140].

Gene	13-HPODE	LA	H_2O_2
PLIN2	$\uparrow\uparrow$ ³	$\uparrow ^{2}$	N ¹
HMGCS2	$\uparrow\uparrow\uparrow$ 3	\uparrow	$\uparrow \uparrow$
CPT1A	$\uparrow\uparrow$	\uparrow	n
FABP1	$\uparrow\uparrow$	$\uparrow\uparrow$	\uparrow
PCK1	$\uparrow\uparrow\uparrow$	$\uparrow\uparrow$	\uparrow
CREB3L3	\uparrow	n	n
DDIT4	$\uparrow\uparrow$	$\uparrow\uparrow\uparrow$	\uparrow
AKR1C3	$\uparrow\uparrow$	\uparrow	\uparrow
PEX6	$\uparrow\uparrow$	$\uparrow\uparrow$	\uparrow
CAT	$\uparrow\uparrow$	\uparrow	$\uparrow \uparrow$
CROT	$\uparrow\uparrow$	\uparrow	$\uparrow\uparrow$
TTN	$\uparrow\uparrow$	\uparrow	\uparrow
RGCC	$\downarrow \downarrow ^{3}$	\downarrow ²	\downarrow
ODC1	$\downarrow\downarrow$	$\downarrow\downarrow$	\downarrow
COX20	\downarrow	\downarrow	\downarrow
CXCL1	$\downarrow\downarrow$	$\downarrow\downarrow$	\downarrow
IL18	\downarrow	\downarrow	\downarrow

Table 4. Comparative gene expression. Comparison of expression of selected genes between cells treated with peroxidized linoleic acid (13-HPODE), non-peroxidized linoleic acid (LA), or hydrogen peroxide (H_2O_2) treatments (\uparrow upregulated; \downarrow downregulated).

Gene	13-HPODE	LA	H ₂ O ₂
STX7	\downarrow	$\downarrow\downarrow$	\downarrow
GBE1	$\uparrow\uparrow$	$\uparrow\uparrow$	\uparrow
CHAC1	$\uparrow\uparrow$	$\uparrow\uparrow\uparrow$	\uparrow
CEBPB	$\uparrow\uparrow$	$\uparrow\uparrow\uparrow$	\uparrow
DUSP4	\downarrow	$\downarrow\downarrow$	$\downarrow \downarrow \downarrow ^{3}$
ACOX2	\uparrow	\uparrow	n
BMF	$\uparrow\uparrow$	$\uparrow\uparrow$	\uparrow
BCL2L1	\downarrow	\downarrow	$\downarrow\downarrow$
UGT2B4	$\uparrow\uparrow$	$\uparrow\uparrow$	\uparrow
GSTA2	↑	n	$\uparrow\uparrow$
GSTA4	n	n	\uparrow
ADH6	n	n	1
NQO1	\uparrow	n	n
DKK1	$\downarrow\downarrow$	$\downarrow\downarrow$	\downarrow

Table 4. Cont.

¹ not differentially expressed gene (adjusted p > 0.05); ² differentially expressed gene (DEG; adjusted p < 0.05); ³ DEG with more pronounced effect compared to the other treatments.

5. Conclusions

PDiff Caco-2 cells could simulate in vivo tumor cells and model intestinal epithelial crypt cells in their proliferative phenotype and lack of brush borders. In this study, we show how PDiff Caco-2 cells respond to peroxidized linoleic acid, 13-HPODE, in terms of differential gene expression, gene ontology, and pathway enrichment, which might reflect the response of in vivo intestinal tumor or crypt cells exposed to dietary LOOHs. Figure 7 summarizes the cellular events and processes altered by each treatment. The stress brought to PDiff cells by 13-HPODE caused the cells to upregulate genes and pathways involved in detoxification as rigorous activation of antioxidant response was required to maintain cellular function and prevent damage. Induction of peroxisomal pathway and cytochrome P450 may, on the one hand, metabolize lipids, detoxify radicals, and maintain redox homeostasis, but on the other hand, can increase ROS production and lipid peroxidation and contribute to the development or progression of disease including gastrointestinal cancer. Moreover, enhanced peroxisomal fatty acid oxidation due to either treatment might favor carcinogenesis. This could propose a mechanism by which 13-HPODE contributes to tumor progression or resistance to anticancer therapy. While induction of PPAR signaling by 13-HPODE promoted FA oxidation and lipid metabolism and might have anti-inflammatory and anti-proliferative effects on PDiff Caco-2 cells, it may maintain the tumorigenic phenotype of the cells. Moreover, upregulated glycogen metabolism by either 13-HPODE or LA and upregulated amino acid metabolism by 13-HPODE or H₂O₂ may promote cell survival. Although our results, in part, indicate reduced proliferation, the overall changes in cellular response to 13-HPODE and H₂O₂ may provide a carcinogenic environment. The negative impact we observed of 13-HPODE on electron transport chain and phago-lysosomal degradation pathways might link LOOHs to different intestinal pathologies including IBD and cancer, which could be initiated by intestinal crypt cells. Compared to 13-HPODE, H₂O₂ treatment not only enhanced antioxidant adaptive response, which protects against cytotoxicity, but also upregulated steroid biosynthesis, which is a hallmark of tumor development. This study expands the knowledge of how PDiff Caco-2 cells respond to 13-HPODE, which possibly resembles the response of in vivo intestinal tumor or epithelial crypt cells exposed to dietary LOOHs. In this context, our results suggest that dietary LOOHs, such as 13-HPODE, could trigger a strong antioxidant mechanism in intestinal tumor or epithelial crypt cells, which provides a defensive response to stress. Our results also indicate that dietary LOOHs may modify metabolic processes and alter energy production and degradation processes creating an environment that renders these proliferative cells to initiate or progress disease. Hence, this study provides possible mechanisms by which dietary LOOHs might contribute to disease development or



progression in intestinal crypt or cancer cells. The hypotheses generated by our RNA-seq study can be further investigated using directed biomolecular experiments.

Figure 7. A summary of the cellular events and processes altered in PDiff Caco-2 cells by 13-HPODE, LA, or H₂O₂.

6. Limitations and Future Work

In this study, we carried out our experiments on culture cells, and we used one concentration of treatment-100 μ M, which, in our previous studies, has demonstrated significant differences between treated and untreated cells. Future transcriptomic studies might be dose-dependent and include in vivo experiments as well as testing other products of lipid peroxidation.

Supplementary Materials: Supplementary materials can be accessed at: https://www.mdpi.com/ 2076-3417/11/6/2678/s1. Figure S1. Leucomethylene blue (LMB) assay. Figure S2: Principal component analysis (PCA), Figure S3: Sample-to-sample distance heatmaps generated by DESeq2, Figure S4: MA plots of differentially expressed genes (DEGs) between untreated and treated PDiff Caco-2 cells, Figure S5: Quantitative RT-PCR validation of RNA-seq data in 13-HPODE-treated PDiff Caco-2 cells, Figure S6: Quantitative RT-PCR validation of RNA-seq data in LA-treated PDiff Caco-2 cells, Figure S7: Quantitative RT-PCR validation of RNA-seq data in H₂O₂-treated PDiff Caco-2 cells, Table S1: Primer sequences, Table S2: Number of sequence reads per sample after trimming, Table S3: Differential gene expression in 13-HPODE-treated cells, Table S4: Gene Ontology analysis (biological processes) in 13-HPODE-treated PDiff Caco-2 cells, Table S5: Gene Ontology analysis (molecular functions) in 13-HPODE-treated PDiff Caco-2 cells, Table S6: Gene Ontology analysis (cellular components) in 13-HPODE-treated PDiff Caco-2 cells, Table S7: Differential gene expression in LA-treated cells, Table S8: Gene Ontology analysis (biological processes) in LA-treated PDiff Caco-2 cells, Table S9: Gene Ontology analysis (molecular functions) in LA-treated PDiff Caco-2 cells, Table S10: Gene Ontology analysis (cellular components) in LA-treated PDiff Caco-2 cells, Table S11: Differential gene expression in H₂O₂-treated cells, Table S12: Gene Ontology analysis (biological processes) in H2O2-treated PDiff Caco-2 cells, Table S13: Gene Ontology analysis (molecular functions) in H₂O₂-treated PDiff Caco-2 cells, Table S14: Gene Ontology analysis (cellular components) in H2O2-treated PDiff Caco-2 cells.

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