Alpha-Chlorofatty Acid Accumulates in Activated Monocytes and Causes Apoptosis Through Reactive Oxygen Species Production and Endoplasmic Reticulum Stress

Wen-yi Wang, Carolyn J. Albert, David A. Ford

Objective—Myeloperoxidase-enriched monocytes play important roles in inflammatory disease, such as atherosclerosis. We previously demonstrated that α-chlorofatty aldehydes are produced as a result of plasmalogen targeting by myeloperoxidase-derived hypochlorous acid in activated monocytes. Here, we show α-chlorofatty acid (α-CIFA), a stable metabolite of α-chlorofatty aldehydes, accumulates in activated monocytes and mediates the molecular effects of α-CIFA on monocytes/macrophages.

Approach and Results—Liquid chromatography-mass spectrometry revealed that α-CIFA is elevated 5-fold in phorbol myristate-stimulated human monocytes rising to ≈20 μmol/L when compared with unstimulated cells. Using human THP-1 monocytes and RAW 264.7 cells as in vitro models, we tested the hypothesis that α-CIFA is a cell death mediator that could potentially participate in pathophysiologic roles of monocytes in diseases, such as atherosclerosis. Indeed, 2-chlorohexadecanoic acid, the 16-carbon molecular species of α-CIFA, caused significant apoptosis of primary monocytes. Similarly, 2-chlorohexadecanoic acid also caused apoptosis in THP-1 human monocytes and RAW 264.7 mouse macrophages as determined by annexin V-propidium iodide staining and terminal deoxynucleotidyl transferase dUTP nick end labeling staining, respectively. 2-Chlorohexadecanoic acid treatment also increased caspase-3 activity and poly (ADP-ribose) polymerase cleavage in THP-1 cells. 2-Chlorohexadecanoic acid likely elicits apoptosis by increasing both reactive oxygen species production and endoplasmic reticulum stress because antioxidants and CCAAT/enhancer-binding protein homologous protein block such induced cell apoptosis.

Conclusions—The stable chlorinated lipid, α-CIFA, accumulates in activated primary human monocytes and elicits monocyte apoptosis through increased reactive oxygen species production and endoplasmic reticulum stress, providing a new insight into chlorinated lipids and monocytes in inflammatory disease. *(Arterioscler Thromb Vasc Biol. 2014;34:526-532.)*

Key Words: apoptosis ▪ macrophages ▪ monocytes ▪ peroxidase

Myeloperoxidase-enriched phagocytes are important cellular mediators in many inflammatory diseases, including atherosclerosis.1-4 In response to inflammation, monocytes are recruited to the sites of inflammation and differentiate into macrophages to elicit immune response.4 Apoptosis of monocytes/macrophages is a component of the pathophysiologic sequelae of atherosclerosis.5 Oxidized lipid species have been suggested to be mediators of apoptosis and the progression of atherosclerosis.6,7 However, the role of chlorinated lipids, produced as a result of myeloperoxidase activity, as mediators of apoptosis remains to be explored. Chlorinated lipids are initially produced by the targeting of the vinyl ether bond linking an sn-1 aliphatic group to the glycerol backbone of plasmalogens, resulting in the formation of 2 products: lyso-phospholipid and α-chlorofatty aldehydes (α-CIFALD).2,8,9 It should be noted that plasmalogens are a major lipid subclass found in many mammalian cell types, including endothelial cells, neutrophils, monocytes, smooth muscle cells, and cardiac myocytes. α-CIFALD, including 2-chlorohexadecanal and 2-chloro-octadecanal, are produced in activated neutrophils and monocytes.5,9,10 Furthermore, the concentration of α-CIFALD is elevated in human atherosclerotic lesions and infarcted myocardium.11,12 In neutrophils, α-CIFALD has been shown to be predominantly oxidized to α-chlorofatty acid (α-CIFA), whereas some metabolism of α-CIFALD shown to be reduced to α-chlorofatty alcohol (α-CIFOH).13,14 α-CIFA is elevated in rats treated with lipopolysaccharide and can be further catabolized in hepatocytes through β-oxidation and subsequent α-oxidation, resulting in the production of 2-chloro-octadipic acid (2-CIAa) that is excreted in the urine.15 Although the α-CIFALD, 2-chlorohexadecanal, has been shown to be a potent neutrophil chemoattractant, inhibitor of endothelial nitric oxide synthase, inducer of the expression of cyclooxygenase-2, and mediator of endothelial cell...
2-Chlorofatty Acid and Monocyte Apoptosis

2-ClFA and 2-Chloroocdecanoic Acid Increase Significantly in Primary Human Monocytes on Phorbol Myristate Acetate Stimulation

Our previous study showed that 2-chlorohexadecanal and 2-chlorooctadecanol increase to levels of 3 to 6 pmol/10^6 phorbol myristate acetate–activated primary human monocytes. In these studies, we tested whether endogenously produced 2-ClFALD is metabolized to 2-ClFIA and 2-ClFOH. To determine the levels of both 2-ClFIA and 2-ClFOH in human primary monocytes, the cells were stimulated with 300 nmol/L phorbol myristate acetate for 1 hour. Cellular lipids were extracted and quantitatively analyzed by liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry for 2-ClFIA and 2-ClFOH, respectively. As expected, the total amount of 2-ClFIA and 2-chlorooctadecanoic acid was elevated 5-fold (rising to ≈20 μmol/L) when compared with unstimulated cells (Figure 1A). For 2-ClFOH levels, only 2-chlorohexadecanol was found to increase significantly when compared with unstimulated cells, whereas 2-chlorooctadecanol was undetectable (Figure 1B). Notably, the 2-ClFIA amount is much more than 2-ClFOH, indicating that 2-ClFALD is mainly metabolized to 2-ClFIA instead of 2-ClFOH in human monocytes.

2-ClPA Increases Caspase-3 Activity, Poly (ADP-Ribose) Polymerase Cleavage, and CCAAT/Enhancer-Binding Protein Homologous Protein Expression

Caspase-3 activity was evaluated to examine 2-ClPA–induced apoptosis further. Indeed, 2-ClPA treatment resulted in increased caspase-3 activity in both THP-1 monocytes and RAW 264.7 macrophages (Figure 3A; Figure 1B in the online-only Data Supplement).

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
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<tr>
<td>2-ClFIA</td>
<td>2-chlorohexadecanoic acid</td>
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<td>α-ClFALD</td>
<td>α-chlorofatty aldehyde</td>
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<tr>
<td>α-ClFIA</td>
<td>α-chlorofatty acid</td>
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<tr>
<td>α-ClFOH</td>
<td>α-chlorofatty alcohol</td>
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<tr>
<td>CHOP</td>
<td>CCAAT/enhancer-binding protein homologous protein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

2-ClPA Induces Apoptosis in Primary Human Monocytes, THP-1 Monocytes, and RAW 264.7 Macrophages

Human primary monocytes were treated with various concentrations of 2-ClPA for 3 hours, and apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling staining. 2-ClPA significantly increased apoptotic cell numbers when compared with either blank or HA group (Figure 2A). Importantly, significant apoptosis was observed in primary human monocytes at physiological concentrations of 2-ClPA found in activated human monocytes. The proapoptotic effect of 2-ClPA was then tested on a human monocytic cell line, THP-1 cells. The cells were treated with 2-ClPA for 6 hours, and apoptosis was subsequently examined by annexin V-propidium iodide double staining followed by flow cytometry. Consistent with the findings in primary human monocytes, 2-ClPA increased apoptosis in THP-1 cells when compared with either blank or HA group (Figure 2B). Under all conditions of treatments of THP-1 cells with 2-ClPA, the intracellular 2-ClPA concentration did not exceed the treatment of extracellular concentration (eg, treatments for 18 hours with 10 and 50 μmol/L 2-ClPA resulted in intracellular concentrations of 7.2±1.4 and 45.6±4.9 μmol/L, respectively; and treatments for 1 hour with 10 and 50 μmol/L 2-ClPA resulted in intracellular concentrations of 0.8±0.1 and 14.2±1.1 μmol/L). 2-ClPA-elicited apoptosis was further confirmed in RAW 264.7 macrophages by terminal deoxynucleotidyl transferase dUTP nick end labeling staining, which further demonstrated the disparate proapoptotic effect of 2-ClPA when compared with HA (Figure 1A in the online-only Data Supplement).
Furthermore, poly (ADP-ribose) poly-merase (PARP) cleavage was determined to confirm 2-ClHA–mediated cell apoptosis. 2-ClHA induces a concentration and time-dependent PARP cleavage (Figure 3B and 3C). In addition, CCAAT/enhancer-binding protein homologous protein (CHOP), also known as GADD153, which is a proapoptotic transcription factor activated by ER stress, was evaluated. In THP-1 monocytes, CHOP was present at undetectable or at low levels in untreated or HA-treated groups, whereas 50 μmol/L of 2-ClHA induced a significant expression of CHOP at both mRNA level and protein level, as measured by quantitative real-time polymerase chain reaction and Western blots, respectively (Figure 3B and 3C; Figure II in the online-only Data Supplement). CHOP expression at the mRNA level in RAW 264.7 macrophages was also significantly upregulated by 2-ClHA as determined by quantitative real-time polymerase chain reaction (Figure II in the online-only Data Supplement).

**2-ClHA Initiates an ER Stress Response**

Because the induction of CHOP expression indicated ER stress might be involved in 2-ClHA–induced apoptosis, we further interrogated this possibility. 2-ClHA induced X-box binding protein-1 mRNA splicing in both THP-1 and RAW 264.7 cells as determined by real-time polymerase chain reaction (Figure 4A; Figure III in the online-only Data Supplement). Furthermore, 2-ClHA significantly increased the phosphorylation of eukaryotic initiation factor (eIF)2α without changing total eIF2α expression (Figure 4B). In concert with eIF2α phosphorylation, the target gene of eIF2α, activating transcription factor-4, was upregulated. In addition, glucose-regulated
protein-78, which serves as a gatekeeper to the activation of ER stress transducers, was increased in response to 2-ClHA treatment (Figure 4B). Other data showed that mRNA expression of these ER stress-associated proteins in THP-1 monocytes or RAW 264.7 cells was increased in response to 2-ClHA (Figure II in the online-only Data Supplement).

CHOP Silencing Attenuates 2-ClHA–Induced Apoptosis
To assess ER stress involvement in 2-ClHA–induced apoptosis further, the effector gene of ER stress, CHOP, was knocked down by its specific siRNA. Transfection of CHOP siRNA effectively reduced CHOP expression, whereas the control siRNA had no such effect (Figure 5A). CHOP silencing resulted in reduced PARP cleavage in response to 2-ClHA (Figure 5A). Furthermore, CHOP silencing significantly decreased caspase-3 activity induced by 2-ClHA (Figure 5B).

ROS Is Involved in 2-ClHA–Induced Apoptosis
A previous study has shown that hypochlorite-modified low-density lipoprotein (LDL)–induced apoptosis is mediated by ROS in Jurkat T cells.\textsuperscript{19} Because LDL contains plasmalogens,\textsuperscript{20} it is likely that one component of hypochlorite-modified LDL is 2-chlorohexadecan-9-ol that can be oxidized intracellularly to 2-ClHA. Accordingly, we investigated whether 2-ClHA alone is sufficient to induce ROS generation.

Figure 3. 2-Chlorohexadecanoic acid (2-ClHA) increases caspase-3 activity and induces poly (ADP-ribose) polymerase (PARP) cleavage and CCAAT/enhancer-binding protein homologous protein (CHOP) expression. A, Caspase-3 activity in THP-1 monocytes treated with indicated concentrations of HA or 2-ClHA for 6 hours was determined as described in Materials and Methods in the online-only Data Supplement. B, THP-1 monocytes were treated with indicated concentrations of 2-ClHA for 18 hours, and PARP cleavage and CHOP expression were determined by Western blots. C, THP-1 monocytes were treated with 50 μmol/L of 2-ClHA for indicated time, and PARP cleavage and CHOP expression were determined by Western blots. Numbers under the blots represent the ratio of the intensity of the response to that of the maximum response in that blot with normalization to the β-actin–loading control, and values are the average of ratios from 3 independent experiments. **P<0.01 vs blank.

Figure 4. 2-Chlorohexadecanoic acid (2-ClHA) induces endoplasmic reticulum stress response. A, THP-1 cells were treated with HA or 2-ClHA for 18 hours, and X-box binding protein-1 mRNA splicing was determined by quantitative real-time polymerase chain reaction. B, THP-1 cells were treated with 50 μmol/L of 2-ClHA for 6 or 18 hours, the expression of phospho-eukaryotic initiation factor (eIF)2α, total eIF2α, activating transcription factor (ATF)4, and glucose-regulated protein-78 was determined by Western blotting. Numbers under the blots represent the ratio of the intensity of the response to that of the response in the blank (control) condition, and values are the average of ratios from 3 independent experiments. In addition, fold induction of phospho-eIF2α was normalized to total eIF2α, whereas the other proteins were normalized to β-actin. S indicates spliced; and u, unspliced.

Figure 5. CHOP silencing attenuates 2-ClHA–induced apoptosis. A, Standard curves of CHOP expression in THP-1 cells treated with HA or 2-ClHA for indicated time. Numbers under the blots represent the ratio of the intensity of the response to that of the maximum response in that blot with normalization to the β-actin–loading control, and values are the average of ratios from 3 independent experiments.

Figure 6. 2-Chlorohexadecanoic acid (2-ClHA) induces ROS production. A, Dihydroethidium (DHE) staining and measurement of ROS production in THP-1 cells treated with HA or 2-ClHA for indicated time. B, Tetramethylrhodamine, ethyl ester (TMR) staining and measurement of ROS production in THP-1 cells treated with HA or 2-ClHA for indicated time.
Amplex Red was used to show that 2-ClHA treatments lead to H$_2$O$_2$ release from THP-1 cells (Figure 6A). In addition, both N-acetylcysteine and glutathione ameliorated the production of H$_2$O$_2$ induced by 2-ClHA. Moreover, N-acetyl cysteine and glutathione attenuated 2-ClHA–induced caspase-3 activity, PARP cleavage, and CHOP expression (Figure 6B and 6C). Thus, these results suggest that ROS might mediate 2-ClHA–induced ER stress.

**Discussion**

The present study shows for the first time that human monocyte activation results in α-CIFA and α-CIFOH production. The production of these chlorinated lipids has previously been shown in other systems to be a result of plasmalogen oxidation by hypochlorous acid leading to α-CIFALD accumulation that is subsequently metabolized through the fatty acid–fatty alcohol cycle. It should also be appreciated that the present studies demonstrate that intracellular levels of α-CIFA reach ≈20 μmol/L during monocyte activation. Furthermore, because activation and apoptosis of monocytes are key events in the formation and rupture of atherosclerotic plaques, the role of this lipid in monocyte apoptosis was examined. Multiple lines of evidence showed that this concentration of α-CIFA elicits monocyte/macrophage apoptosis, including increased terminal apoptosis.

**Figure 5.** Effect of CCAAT/enhancer-binding protein homologous protein (CHOP) siRNA on 2-chlorohexadecanoic acid (2-ClHA)–induced apoptosis. THP-1 monocytes transfected with scrambled siRNA or CHOP siRNA were treated with 50 μmol/L of 2-ClHA, then poly (ADP-ribose) polymerase (PARP) cleavage, as well as CHOP expression (18-hour treatment) was determined by Western blots (A) and caspase-3 activity (6-hour treatment) was measured as described in Materials and Methods in the online-only Data Supplement. B, Numbers under the blots represent the ratio of the intensity of the response to that of the maximum response in that blot with normalization to the β-actin–loading control, and values are the average of ratios from 3 independent experiments. **P<0.01 between treatments that included the addition of 2-ClHA.

**Figure 6.** Reactive oxygen species (ROS) is involved in 2-chlorohexadecanoic acid (2-ClHA)–induced apoptosis. THP-1 monocytes were pretreated or not with the antioxidants, N-acetylcysteine (NAC; 1 mmol/L) or glutathione (GSH; 1 mmol/L) for 1 hour, and then with 50 μmol/L of 2-ClHA for 3 hours (A), 6 hours (B), or 18 hours (C). Extracellular H$_2$O$_2$ levels (A) and caspase-3 activity (B) were determined as described in the Materials and Methods in the online-only Data Supplement. n=3. **P<0.01 vs blank. ##P<0.01 vs 2-ClHA. Poly (ADP-ribose) polymerase (PARP) cleavage and CCAAT/enhancer-binding protein homologous protein (CHOP) expression (C) were determined by Western blotting. Numbers under the blots represent the ratio of the intensity of the response to that of the maximum response in that blot with normalization to the β-actin–loading control, and values are the average of ratios from 3 independent experiments.
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deyxynucleotidyl transferase dUTP nick end labeling staining, annexin V-propidium iodide staining, and caspase-3 activity in primary human monocytes, as well as increased levels of these apoptotic measures and PARP cleavage in both THP-1 monocytes and RAW 264.7 macrophages. Interestingly, a previous study indicated hypochlorite-modified LDL caused cell death in Jurkat T-cell lines. Such modified LDL is likely to contain α-CIFALD, which can be further oxidized to α-CIFA intracellularly. Although there are many chlorinated moieties in hypochlorite-modified LDL, including tyrosine and cysteine residues, it is possible that α-CIFALD residues in the modified LDL may elicit at least, in part, the injury observed in cells treated with hypochlorite-treated LDL.

ER stress activation is frequently observed in atherosclerotic lesions, liver tissue, and adipose tissue of hyperlipidemic mice and humans. Lipid accumulation has been suggested to activate ER stress. Extracellular exposure to high levels of saturated fatty acids induces ER stress and apoptosis in liver cells. Stearic acid accumulation in macrophages results in ER stress, as well as apoptosis independent of toll-like receptor 4/2 activation. The present study is the first to report that chlorinated lipids can also induce ER stress in monocytes/macrophages, which leads to increased CHOP expression. In fact, 2-CIHA seems to be more potent in activating ER stress than HA because HA did not initiate ER stress or apoptosis at the equimolar concentrations. The role of ER stress in CIHA-elicited apoptosis was strongly supported by the demonstration that reducing CHOP expression with its specific siRNA significantly decreases 2-CIHA–induced apoptosis in THP-1 monocytes.

The finding that 2-CIHA upregulates CHOP expression indicates that ER stress may mediate 2-CIHA’s effects. Two of the 3 major ER stress pathways are inositol-requiring enzyme-1 and RNA-dependent protein kinase–like ER kinase, and these 2 pathways were involved in 2-CIHA–elicited ER stress. Inositol-requiring enzyme-1 triggers the splicing of a specific mRNA transcript for X-box binding protein-1, and activation of protein kinase–like ER kinase phosphorylates eIF2α, which lead to the transcription of CHOP and GRP78. The possible mechanism of CHOP-induced apoptosis involves interaction with members of the B-cell lymphoma 2 (BCL-2) family of proteins or a calcium signaling pathway. Previous studies have shown that ER stress signaling can contribute to apoptotic cell death in several cell types related to the cardiovascular system. In vitro studies suggested increased CHOP expression in apoptotic smooth muscle cells treated with 7-ketocholesterol, homocysteine, or glucosamine. Also, the activation of ER stress has been identified in endothelial cells both in vitro and in swine.

Our studies also suggest that ROS are also involved in increasing ER stress and apoptosis. In the present study, we demonstrated that 2-CIHA can induce ROS production using Amplex Red to monitor extracellular H2O2 production. In addition, the antioxidants, N-acetyl cysteine and glutathione, decreased 2-CIHA–induced ROS production and apoptosis. Furthermore, N-acetyl cysteine and glutathione attenuated CHOP expression, indicating a mitigation of ER stress. Thus, ROS generation may mediate 2-CIHA–triggered ER stress. It should be noted that the interplay between ROS and ER stress has been reported in many studies although the detailed mechanism is still elusive. Attenuation of ER stress decreases ROS generation and increases glutathione level in α-tocopheryl succinate–treated human gastric carcinoma cells. Some other studies have reported that oxidative stress induces ER stress in cultured human hepatoma cells and hepatocytes.

In summary, the results of this study demonstrate that α-CIFA accumulates in activated monocytes and contributes to apoptosis by triggering ROS production and ER stress. Silencing of CHOP expression attenuates the apoptosis-inducing effect of 2-CIHA, whereas attenuation of ROS by antioxidants can alleviate ER stress and subsequent cell apoptosis. Thus, our study suggests that α-CIFA is a bioactive lipid that links inflammation and apoptosis in monocytes/macrophages. This is an important first step, albeit in isolated cell systems, to show the biological role of α-CIFA, which may be of considerable importance in a modulatory role in some inflammatory diseases, such as atherosclerosis. Demonstrating the role of both exogenously applied, as well as endogenously produced, α-CIFA in vivo systems of inflammation, including atherosclerosis, needs to be examined in future studies.

Sources of Funding

This research was supported by National Institutes of Health grants HL074214 and HL111906 (D.A. Ford).

Disclosures

None.

References

21. α-roles in inflammation and cardiovascular diseases. This study demonstrates (1) for the first time, micromolar levels of α-chlorofatty acid are increasing evidence has suggested that chlorinated lipids produced from the myeloperoxidase-hypochlorous acid system may play important roles in inflammation and cardiovascular diseases. This study demonstrates (1) for the first time, micromolar levels of α-chlorofatty acid are produced in activated human monocytes; (2) α-chlorofatty acid initiates reactive oxygen species production and subsequent endoplasmic reticulum stress leading to monocyte death; and (3) α-chlorofatty acid initiated apoptosis is reduced by either antioxidant treatment or downregulation of CCAAT/enhancer-binding protein homologous protein. Thus, the production of α-chlorofatty acid is a novel mechanism that potentially links inflammation and cell death, and, in particular, this pathway may be involved in the inflammation, apoptosis, and endoplasmic reticulum stress that occurs in atherosclerotic lesions.

Significance

Increasing evidence has suggested that chlorinated lipids produced from the myeloperoxidase-hypochlorous acid system may play important roles in inflammation and cardiovascular diseases. This study demonstrates (1) for the first time, micromolar levels of α-chlorofatty acid are produced in activated human monocytes; (2) α-chlorofatty acid initiates reactive oxygen species production and subsequent endoplasmic reticulum stress leading to monocyte death; and (3) α-chlorofatty acid initiated apoptosis is reduced by either antioxidant treatment or downregulation of CCAAT/enhancer-binding protein homologous protein. Thus, the production of α-chlorofatty acid is a novel mechanism that potentially links inflammation and cell death, and, in particular, this pathway may be involved in the inflammation, apoptosis, and endoplasmic reticulum stress that occurs in atherosclerotic lesions.


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Arterioscler Thromb Vasc Biol. 2014;34:526-532; originally published online December 26, 2013;
doi: 10.1161/ATVBAHA.113.302544

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL FIGURE LEGENDS

Figure SI. 2-CIHA induces apoptosis of RAW 264.7 macrophages. A. Cells were treated with indicated concentrations of HA or 2-CIPA for 24h, and the apoptotic cells were determined under fluorescent microscopy after TUNNEL staining. B. Caspase 3 activity in cells treated with indicated concentrations of HA or 2-CIHA for 24h. N=3. *P<0.05 and **P<0.01 vs. Blank.

Figure SII. 2-CIHA induces mRNA expression of ER stress-associated proteins. THP-1 cells (A) or RAW 264.7 cells (B) were treated with 50 µM of HA or 2-CIHA for 18h, and the mRNA levels of indicated proteins were determined by qRT-PCR. N=3. **P<0.01.

Figure SIII. 2-CIHA induces XBP1 mRNA splicing in RAW 264.7 cells. Cells were treated with 50 µM of HA or 2-CIHA for 18h, and XBP1 mRNA splicing was determined by qRT-PCR.

Table SI. Primer sequences

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8 for realtime PCR, 9 for XBP1.

REFERENCES


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Figure SIII. 2-CIHA induces XBP1 mRNA splicing in RAW 264.7 cells. Cells were treated with 50 μM of HA or 2-CIHA for 18h, and XBP1 mRNA splicing was determined by qRT-PCR.
MATERIALS AND METHODS

Reagents
Palmitic acid (HA) was purchased from NuChek Prep. 2-ClHA, 2-chloro-[d_7,7,8,8]-hexadecanoic acid (2-Cl-[d_7]HA), and 2-chloro-[d_7,7,8,8]-hexadecanol (2-Cl-[d_7]HOH) were synthesized as previously described. Other reagents, unless specified, were purchased from Sigma-Aldrich (Saint Louis, MO).

Primary human monocytes isolation and activation
Human monocytes were isolated from peripheral blood as described previously. In brief, anticoagulated blood was diluted with Ca^{2+}/Mg^{2+}-free HBSS and subjected to Ficoll density gradient centrifugation (density, 1.077 g/ml; 400 xg for 45 min). The middle layer of cells containing monocytes were collected and diluted 1:1 with Ca^{2+}/Mg^{2+}-free HBSS, which was then subjected to further Ficoll density gradient centrifugation (density, 1.070 g/ml; 400 xg for 15 min). The mid-floating layer was plated on 60-mm culture dishes in Medium 199 and cultured at 37 °C for 1 h in the presence of 5% CO_2. Before the experiments, monocytes were washed three times in fresh HBSS. These cultured human monocytes were found to be void of granulocytes as determined by differential staining. Monocytes were then incubated in 2 ml of HBSS containing Mg^{2+} and Ca^{2+} at 37°C for 30 min or 1 h in the presence and absence of phorbol myristate acetate (PMA) (300 nM). At indicated time points, cells were scraped into 1.6 ml of ice-cold saline on ice and stocked at -80°C until lipid extraction.

Lipid extraction and analysis
Cellular lipids were extracted by conventional liquid-liquid extraction. For each sample, 20 pmol of 2-Cl-[d_7]HOH and 2-Cl-[d_7]HA were added as internal standards for 2-ClHOH and 2-ClHA quantification, respectively. The final lipid extract was resuspended in 1 ml of chloroform, and then 0.1 ml and 0.4 ml sample were taken for α-ClFA and α-ClFOH analysis, respectively. For free α-ClFA analysis, the samples in chloroform were dried under nitrogen and resuspended in 200µl of methanol/water (85/15, v/v) containing 0.1% formic acid. After vigorous vortexing, the samples were transferred to an autosampler tube with an insert for analysis by liquid chromatography-mass spectrometry (MS) following previously described methods. Both cellular α-ClFA and α-ClFOH were expressed in µM based on the conversion of 0.334µl cell volume in 10^6 monocytes.

α-ClFOH was first derivatized to its pentafluorobenzoyl ester before quantitation by gas chromatography-MS. The samples in chloroform were dried under nitrogen and 50µl of pentafluorobenzyl chloride was added. Then, the samples were capped under nitrogen and incubated at 60°C for 45 min. After incubation, the reaction was cooled down to room temperature for 10 min, centrifuged at 400 xg for 2 min, and dried under nitrogen. The remaining derivatized products were washed twice with 2ml of hexane, resuspended in 300µl of hexane and transferred to the autosampler vial insert. After solvent was evaporated under nitrogen, the samples were resuspended in 100µl of ethyl acetate before being subjected to gas chromatography-MS analyses as previously described.

Cell culture
Isolated primary human monocytes were washed three times in fresh HBSS and cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) overnight for apoptosis assay. Before the experiments, cells were washed with the medium once and then treated with indicated concentrations of hexadecanoic acid (HA) or 2-CIHA for 3h in RPMI 1640 containing 2% FBS. Human monocytic THP-1 cells were grown in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS. For experiments, THP-1 cells were seeded in 35-mm dishes with a density of 7.5×10^5 cells/mL and cultured in 2% FBS-containing fresh medium. Mouse macrophage RAW 264.7 cells were cultured in DMEM medium (Sigma-Aldrich) containing 10% FBS. Before experiments, fresh medium containing 2% FBS was added.

Cell apoptosis analysis
For THP-1 monocytes, cell apoptosis was measured by the Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit (V13245, Invitrogen Inc.). Cells were harvested and washed twice with cold PBS. Cell pellets were resuspended in 1x Annexin V-binding buffer containing Annexin V and propidium iodide (PI). After incubation at room temperature in the dark for 15 min, the samples were diluted to 500 µL with 1x annexin-binding buffer, and analyzed by flow cytometry measuring the fluorescence emission at 530 nm and 575 nm using 488 nm excitation.

For primary human monocytes and RAW 264.7 cells, apoptotic cells were determined by an in situ cell death detection kit from Roche. Cells were washed once with PBS and then fixed in 4% paraformaldehyde for 1h. After washing with PBS, the samples were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min and labeled with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture as well as 4’,6’-diamidino-2-phenylindole. Finally, the samples were washed with PBS twice and evaluated by fluorescence microscopy.

Caspase 3 activity assay
Caspase 3 activity in cell lysates was measured by a fluorometric EnzChek® Caspase 3 Assay Kit (Cat. E13183) from Invitrogen Inc. Briefly, cells were washed in cold PBS twice and then resuspended in 1x cell lysis buffer for protein extraction. The protein concentration of the samples was determined using a Bio-Rad protein assay kit. 50 µl of samples and 50 µl of 2x reaction buffer containing 200 µM of substrate N-Benzoyl-DEVD-amino-4-methylcoumarin were added in a black 96-well plate with flat bottom (FALCON Inc.) and incubated at 37°C in a plate reader (BIO TEK Inc.) for monitoring the fluorescence using 342/441 nm excitation/emission wavelengths at multiple time points up to 1h. The rate of fluorescence increase indicates the caspase 3 activity in each sample. Finally, the caspase 3 activity of the fluorescence increase was normalized to the sample protein content.

Western blots
Cells were washed once with cold PBS and lysed in RIPA buffer (Pierce, Lot. 89900) containing protease inhibitor cocktail (Roche). After incubation for 15 minutes on ice, the samples were centrifuged at 14,000g for 15 min at 4°C, and the supernatant was collected as whole cell lysate and stored at -20°C until use. Equivalent amounts of protein were separated
on SDS-PAGE and transferred to polyvinylidene fluoride membranes. The following primary antibodies were used: cleaved poly (ADP-ribose) polymerase (PARP, Cat.556362, BD Pharmingen), CHOP (sc-7351, Santa Cruz), GRP78 (sc-13968), ATF4 (sc-200), eIF2α (Cat.5324, Cell Signaling) and phospho-eIF2α (Cat.9721, Cell Signaling). The antibody of β-actin (A3854, Sigma) was used as the loading control.

**RNA isolation and analysis**

Total RNA was isolated using TRIZOL reagent (Invitrogen Inc). After DNase treatment, 1 µg of RNA was reverse transcribed to cDNA using Taqman First Strand Synthesis kit (Invitrogen) with oligo-dT priming. Quantitative realtime PCR (qRT-PCR) was performed in triplicate in a final volume of 10 µl using SYBR green PCR master mix (Applied Biosystems) on Roche 480 lightcycle instrument with the following amplification conditions: 95°C for 10 min, 45 cycles at 95°C for 15s and 60°C for 30s. At the end of each run, a melting curve analysis from 55°C to 90°C was performed to ensure the absence of non-specific products and primer dimers. Glyceraldehyde phosphate dehydrogenase (GAPDH) and β-actin were used as control genes for human and mouse gene expression, respectively, with the following conditions: 95°C for 5 min, 32 cycles at 94°C for 30s, 53°C for 30s and 72°C for 30s and a final extension step at 72°C for 5 min.

For detection of Xbp-1 mRNA splicing, PCR amplification of cDNA prepared as described above was performed using REDTaq ReadyMix PCR Reaction Mix (Sigma) with the following conditions: 95°C for 5 min, 32 cycles at 94°C for 30s, 53°C for 30s and 72°C for 30s and a final extension step at 72°C for 5 min. The PCR products were separated on 2% agarose (Invitrogen) gel and visualized with ethidium bromide staining. The information of all the primers is provided in Table SI.

**Hydrogen peroxide determination**

Hydrogen peroxide (H₂O₂) in cell culture medium was determined using Amplex Red (90101, Sigma). Cell culture media was collected and centrifuged at 5000 rpm to remove cell debris. A total reaction volume of 100 µL containing 50 µL of media, 50 µM of Amplex Red, and 0.01 mU of horseradish peroxidase (HRP, Sigma) was added in a black 96-well plate with flat bottom. Fluorescence was monitored for up to 30 min using 530/590 nm excitation/emission wavelengths. The relative H₂O₂ levels in samples were evaluated by determining the rate of fluorescence increase.

**Transient transfections**

Negative control siRNA (sc-36868) and human CHOP siRNA (sc-35437) were transfected to THP-1 monocytes using transfection reagent (sc-39528) from Santa Cruz Inc. according to the kit instructions. Cells were cultured in media with the siRNA present for 6 h, and the transfection efficiency was subsequently estimated by observing the fluorescence under microscopy since the control siRNA is fluorescein isothiocyanate-conjugated. Next, the siRNA was removed by replacing with fresh medium containing dimethylsulfoxide or 2-ClHA for another 18 h.

**Statistical analysis**
All data are presented as means ± std. dev. One-way ANOVA followed by Dunnett post-hoc test was used to determine statistical differences between control and experimental groups. P<0.05 was considered statistically significant.

REFERENCES