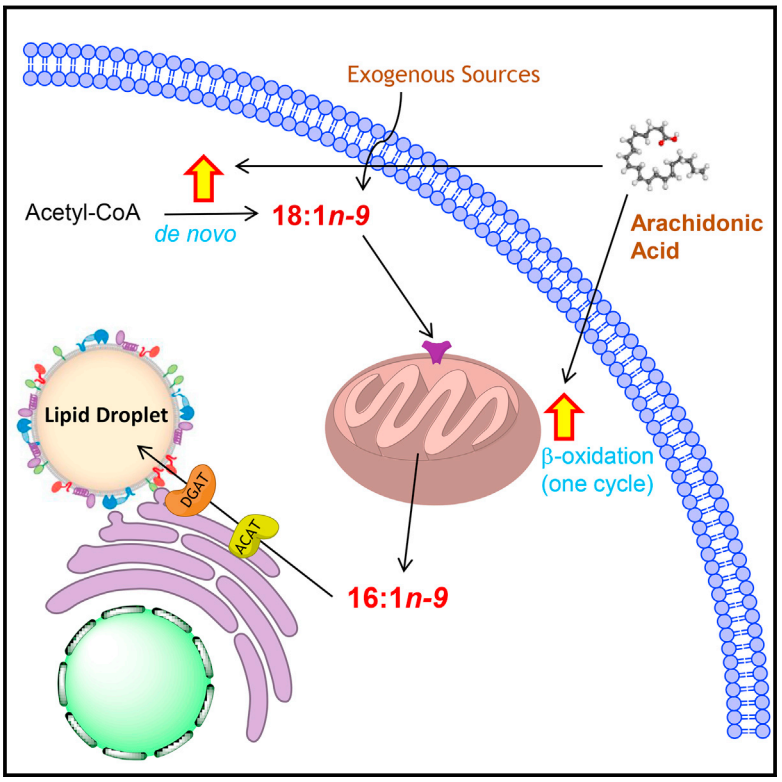
Article

Cell Chemical Biology

Foamy Monocytes Are Enriched in *cis*-7-Hexadecenoic Fatty Acid (16:1n-9), a Possible Biomarker for Early Detection of Cardiovascular Disease

Graphical Abstract



Highlights

- Stimulated monocytes accumulate 16:1n-9, an unusual fatty acid
- Unlike all other fatty acids, 16:1n-9 localizes primarily in neutral lipids
- 16:1n-9 possesses anti-inflammatory properties in vitro and in vivo

Authors

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In Brief

Guijas et al. discovered that phagocytic cells contain *cis*-7-hexadecenoic acid, an unusual isomer of palmitoleic acid, the levels of which are regulated upon activation. The fatty acid shows significant anti-inflammatory activity in vitro and in vivo and may be a biomarker for early detection of cardiovascular disease.

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Foamy Monocytes Are Enriched in *cis*-7-Hexadecenoic Fatty Acid (16:1n-9), a Possible Biomarker for Early Detection of Cardiovascular Disease

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SUMMARY

Human monocytes respond to arachidonic acid, a secretory product of endothelial cells, by activating the de novo pathway of fatty acid biosynthesis, resulting in the acquisition of a foamy phenotype due to accumulation of cytoplasmic lipid droplets. Recruitment of foamy monocytes to endothelium is a key step in the formation of atherosclerotic plaques. Here we describe that lipid droplets of foamy monocytes are enriched in a rather uncommon fatty acid, *cis*-7-hexadecenoic acid (16:1n-9), a positional isomer of palmitoleic acid. 16:1n-9 was found to possess an anti-inflammatory activity both in vitro and in vivo that is comparable with that of omega-3 fatty acids and clearly distinguishable from the effects of palmitoleic acid. Selective accumulation in neutral lipids of phagocytic cells of an uncommon fatty acid reveals an early phenotypic change that may provide a biomarker of proatherogenicity, and a potential target for intervention in the early stages of cardiovascular disease.

INTRODUCTION

Atherosclerosis, a major cause of cardiovascular disease, can be initiated by the increased activation of endothelial cells lining the inside of blood vessels. This activation results in the endothelial cells releasing a variety of products with inflammatory potential that may attract monocytes and favor their infiltration into the subendothelial space (Moore and Tabas, 2011; Tabas and Bornfeldt, 2016). Among the endothelial cell-derived products with capacity to activate monocytes is arachidonic acid (AA; *cis*-5,8,11,14-eicosatetraenoic acid; 20:4n-6) (Østerud and Bjørklid, 2003). In addition to prostaglandins and other oxygenated derivatives of AA, endothelial cells also secrete large amounts of the free fatty acid, which may also act locally to activate neighboring cells (Pérez-Chacón et al., 2009; Astudillo et al., 2012). We have recently shown that exposure of human monocytes to AA results in the cells rapidly accumulating neutral lipids in cytoplasmic droplets (Guijas et al., 2012). Neutral lipid formation occurs subsequent to increased de novo fatty acid formation, and is critically dependent on cytosolic phospholipase $A_2\alpha$ activation (Guijas et al., 2012, 2014).

While the role of circulating monocytes as precursors of macrophages, which after moving to the subendothelial space become foam cells, is clearly defined (Moore and Tabas, 2011; Tabas and Bornfeldt, 2016), our previous results support the emerging paradigm that monocytes interacting with proinflammatory products released by endothelial cells may acquire a foamy phenotype well before they cross the endothelial layer and differentiate into macrophages (Wu et al., 2009; Foster et al., 2015; Robbins et al., 2013; Xu et al., 2015). The accumulation of lipid droplets in the monocytes is accompanied by increased expression of adhesion and chemokine receptors and overall proinflammatory markers, resulting in a higher risk of cardiovascular disease. Thus lipid droplets in circulating monocytes are being considered not only a possible therapeutic target, but also a marker of risk (Wu et al., 2009; Foster et al., 2015; Robbins et al., 2013; Xu et al., 2015). In this regard, our molecular analysis of the fatty acid composition of the neutral lipids of foamy monocytes revealed their selective enrichment in a relatively minor fatty acid, identified as palmitoleic acid (Guijas et al., 2012).

Palmitoleic acid (16:1n-7) has received much interest recently as a promising anti-inflammatory lipid that may help ameliorate metabolic disorders. Work by Cao et al. (2008) introduced the concept of "lipokine" or lipid hormone, which coordinates metabolic responses, to describe the biological actions of 16:1n-7 in mice. The fatty acid, released from the adipose tissue, acted in the liver to suppress steatosis and improve insulin signaling in muscle (Cao et al., 2008). Other studies showed that 16:1n-7 had beneficial effects on muscle cell sensitivity to insulin (Dimopoulos et al., 2006), viability and proliferation of pancreatic β cells (Maedler et al., 2003), and ER stress in macrophages (Erbay et al., 2009). In accordance with the low-grade inflammatory state that is characteristic of obesity and associated metabolic disorders, 16:1n-7 was also shown to reduce the inflammatory response of adipose tissue in certain murine models, and to improve the metabolic responses of liver and muscle by impacting on AMP-activated protein kinase and mitogen-activated protein kinase signaling pathways (Talbot et al., 2014; Chan et al., 2015).

However, studies in human subjects have often produced conflicting results (Hodson and Karpe, 2013; De Fabiani, 2011). Epidemiological studies reported a positive correlation between high circulating 16:1n-7 levels and obesity (Gong et al., 2011), pancreatitis (Samad et al., 2014), hepatic steatosis (Lee et al., 2015), and cardiovascular disease (Warensjo et al., 2008). Other studies showed that sensitivity to insulin in peripheral tissues is not associated with the availability of 16:1n-7 in plasma of obese patients (Fabbrini et al., 2011), and that high levels of this fatty acid in red blood cells correlate with severity of inflammation (Perreault et al., 2014) and greater risk of diabetes (Zong et al., 2013).

By refining and improving our protocol of detection of fatty acids, we describe in this work that human monocytes synthesize, in addition to palmitoleic acid (16:1n-7), an unusual 16:1 fatty acid, *cis*-7-hexadecenoic acid (16:1n-9). Unexpectedly, we have found that 16:1n-9, not 16:1n-7, is the fatty acid that accumulates in the neutral lipids of lipid droplets of monocytes, raising the possibility that 16:1n-9 may constitute a biomarker for foamy monocyte formation. Further evidence is presented that 16:1n-9 acts as a novel anti-inflammatory lipid and participates in a novel lipid "class-switching" phenomenon, attenuating responses induced by proinflammatory stimuli.

RESULTS

Stimulation of Monocytes by AA Induces the Accumulation of an Unusual Fatty Acid in Neutral Lipids

In previous work we used gas chromatography-mass spectrometry (GC-MS) to identify the selective increase of a 16:1 fatty acid in lipid droplets of AA-treated monocytes (Guijas et al., 2012). It was assumed that this fatty acid would be palmitoleic acid (16:1n-7), as this is the only 16:1 isomer regularly identified in studies dealing with fatty acid analysis (Quehenberger et al., 2011). Note that when fatty acid methyl esters (FAMEs) are analyzed by electron impact MS, positional isomers of monounsaturated fatty acids all exhibit identical fragmentation spectra (Christie, 1998). By perfecting our chromatographic protocol to achieve greater separation between palmitic acid (16:0) and stearic acid (18:0), the retention time of the peak initially identified as 16:1 in triacylglycerol (TAG) and cholesteryl ester (CE) (13.22 min) no longer coincided with the retention time of an authentic 16:1n-7 standard (13.29 min) (Figures 1A and 1B). Thus, the 16:1 fatty acid accumulating in neutral lipids of activated monocytes is actually not 16:1n-7 but an unidentified isomer.

We purchased all commercially available 16:1 isomers and compared their retention times under present conditions with that of the unidentified fatty acid. The isomers studied were palmitoleic acid (*cis*-9-hexadecenoic acid, 16:1n-7), *trans*-palmitoleic acid (*trans*-9-hexadecenoic acid, 16:1n-7t), sapienic acid (*cis*-6-hexadecenoic acid, 16:1n-10), palmitvaccenic acid (*cis*-11-hexadecenoic acid, 16:1n-5), and *cis*-7-hexadecenoic acid (16:1n-9). Figure 1A shows the retention times of the five 16:1 isomers, and Figure 1B shows the retention time of the unidentified fatty acid present in the neutral lipid fractions of monocytes. Comparison of both figures shows that the unidentified fatty acid has the same retention time as 16:1n-9 and 16:1n-10, and is clearly distinguished from the other isomers.

Despite many attempts under different conditions we could not resolve 16:1n-9 from 16:1n-10 as FAMEs. Thus we opted for derivatization to dimethyloxazolines (DMOX). Unlike the derivatization to FAMEs, DMOX derivatization allows unambiguous localization by electron impact MS of the double bonds of monounsaturated fatty acids (Zhang et al., 1988; Christie et al., 2000; Fay and Richli, 1991). First, we used commercial standards of 16:1n-9 and 16:1n-10 to fully characterize the DMOX derivatives. These also eluted together under present GC conditions; however, their fragmentation spectra by electron impact MS showed clear differences, with diagnostic peaks of *m/z* 167 for 16:1n-10 (Figure 1C), and *m/z* 168 for 16:1n-9 (Figure 1D). Another diagnostic feature is the relative difference in the intensity of major peaks of *m/z* 113 and 126, which appear with similar intensity for the 16:1n-9 derivative but not for 16:1n-10. Finally, the fragment of *m/z* 194 is prominent for 16:1n-10 but not for 16:1n-9, while the fragment of *m/z* 208 is prominent for 16:1n-9 but not for 16:1n-10.

With this information we proceeded to carry out DMOX derivatizations of the fatty acids present in the neutral lipid fractions (TAG and CE) of AA-treated human monocytes. The fragmentation spectrum of the unidentified 16:1 fatty acid found in the two fractions, as shown in Figure 1E, is basically identical to that of pure 16:1n-9 shown in Figure 1D, namely, fragments of *m/z* 113 and 126 show similar intensities; there is a 12-mass unit difference between fragments of *m/z* 180 and 168, indicative of the existence of a double bond between carbons 7 and 8 (Zhang et al., 1988); and fragment of *m/z* 208 is prominent, whereas fragment of *m/z* 194 is of low intensity (Figures 1E and 1F). The data demonstrate that the fatty acid accumulating in neutral lipids is 16:1n-9.

Localization of 16:1n-9 within Cellular Lipids

In unstimulated monocytes, 16:1n-9 was present primarily in neutral lipids (TAG and CE) but distinct amounts were also detected in phospholipids (Figure 2A). Unlike neutral lipids, analysis of phospholipids revealed two 16:1 peaks, one corresponding to 16:1n-7 and the other to 16:1n-9. Thus, human monocytes contain both the n-7 and n-9 isomers (Figures 2A and 2B), but only the n-9 isomer accumulates in neutral lipids (Figures 2A and 2B). Furthermore, resting human monocytes were found to contain three times as many 16:1n-9 as 16:1n-7 (Figure 2A). The amounts of both isomers as free fatty acids were negligible.

The distribution of other major fatty acids is shown in Figure 2C. Saturated fatty acids (palmitic acid, 16:0; stearic acid, 18:0) were found primarily in phospholipids, and distributed more or less evenly between all classes of neutral lipids. The preference for esterification in phospholipids over neutral lipids was more pronounced for unsaturated fatty acids (oleic acid, 18:1n-9; linoleic acid, 18:2n-6). Finally, polyunsaturates (AA, 20:4n-6; docosahexaenoic acid, 22:6n-3) were found almost exclusively in phospholipids, with very minor amounts in TAG. These data show that the distribution of 16:1n-9 between cellular lipids does not resemble that of any other cellular fatty acid; 16:1n-9 is the only fatty acid that incorporates preferentially in neutral lipids over phospholipids.

Accumulation of 16:1n-9 was also examined in monocytes differentiated toward macrophages with recombinant macrophage colony-stimulating factor (Rubio et al., 2015). While the

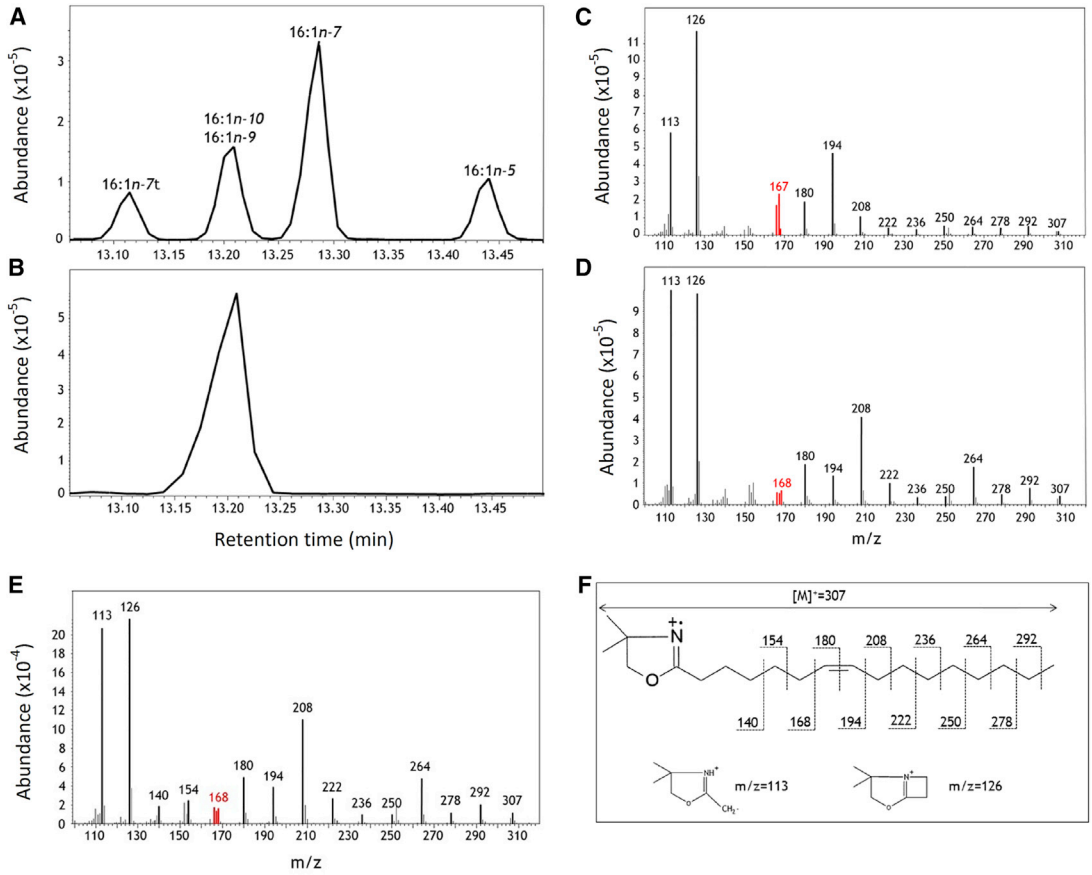

Figure 1. Identification of the 16:1 Isomer Present in Neutral Lipids of Human Monocytes

(A) Retention times of authentic 16:1 fatty acid isomers, as measured by GC-MS.

(B) Retention time of the 16:1 fatty acid present in TAG and CE fractions of human monocytes. The esters of glycerol and cholesterol, derivatized to FAMEs, were measured by GC-MS.

(C) Fragmentation spectrum by electron impact MS of the DMOX derivative of an authentic 16:1n-10 standard. Diagnostic peak at m/z 167 is highlighted in red.

(D) Fragmentation spectrum by electron impact MS of the DMOX derivative of an authentic 16:1n-9 standard. Diagnostic peak at m/z 168 is highlighted in red.

(E) Fragmentation spectrum by electron impact MS of the DMOX derivative of the 16:1 fatty acid present in TAG and CE fractions of human monocytes. Diagnostic peak at m/z 168 is highlighted in red.

(F) Schematic diagram of the fragments generated from the DMOX derivative of 16:1n-9.

level 16:1n-9 greatly increased along the differentiation process, 16:1n-7 content did so to a much lesser extent (Figure S1). These data indicate that also in monocyte-derived macrophages, the content of 16:1n-9 appreciably exceeds that of 16:1n-7.

The relative distribution of 16:1n-9 between lipid classes was also studied after monocyte activation with AA (10 μM, 2 hr) (Guijas et al., 2012). The cellular amount of 16:1n-9 was increased by 3- to 4-fold (5.9 ± 0.8 in resting cells versus 21.3 ± 3.3 nmol/mg protein in activated cells; mean ± SE, n = 5). Interestingly, 16:1n-9 augmented in CE and TAG but not in phospholipids, implying that the increased amounts of 16:1n-9 found in neutral lipids of activated cells do not originate from transfer from phospholipids, but rather that the newly formed 16:1n-9 is directly shuttled to neutral lipids.

Route of Biosynthesis of 16:1n-9

Since mammalian cells do not express Δ^7 desaturases (Guillou et al., 2010), 16:1n-9 cannot be synthesized directly from palmitic acid. 16:1n-9 could be formed via two-carbon elongation of physeteric acid (14:1n-9); however, we have consistently failed to detect even traces of 14:1n-9 in human monocytes or in monocyte-derived macrophages, which contain four to five times as many fatty acids as monocytes (Guijas et al., 2012). A more likely origin of 16:1n-9 could be via β-oxidation of oleic acid (18:1n-9). To study this possibility, we enriched cells with oleic acid by treating them with various amounts of this fatty acid in serum-free media for 16 hr. Consistent with a precursor/product relationship, elevated cellular levels of oleic acid correlated with corresponding increases in cellular 16:1n-9 (Figure 3A). To address this question more directly, we utilized uniformly deuterated oleic acid, where all hydrogen atoms have been replaced by deuterium atoms. Figures 3B and 3C show the mass spectra of the FAMEs of oleic acid and its uniformly deuterated isotopomer, respectively. Note from the comparison of both spectra that the fragments formed after electron impact of oleic acid methyl ester ($[M-32]^+$, $[M-74]^+$, and $[M-116]^+$), which are commonly found in spectra of monounsaturated FAMEs, are not formed in the deuterated variety, where these fragments are

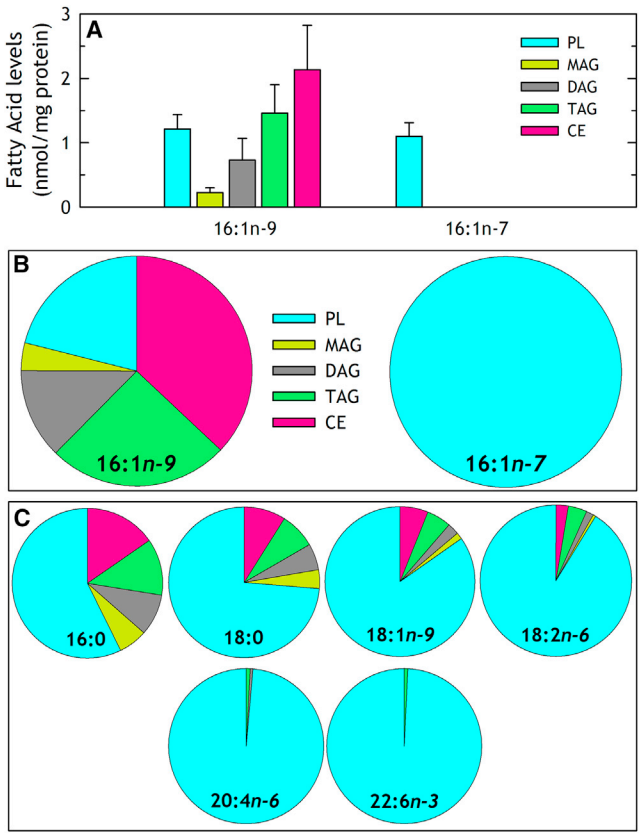

Figure 2. Comparative Distribution of Isomers of Fatty Acid 16:1 in Human Monocytes

(A and B) Distribution by mass (A) and percentage (B) of the two 16:1 isomers present in human monocytes. Data are shown as mean ± SE (n = 4).

(C) Percent distribution of major fatty acids among the various lipid classes in human monocytes. CE, cholesteryl ester; DAG, diacylglycerol; MAG, monoacylglycerol; PL, phospholipid; TAG, triacylglycerol.

See also Figures S1–S3.

replaced by others at *m/z* $[M-33]^+$, $[M-77]^+$, and $[M-125]^+$. Likewise, the major diagnostic fragments of oleic acid at *m/z* 55, 74, and 83 are now found at *m/z* 62, 77, and 94 in the uniformly deuterated isotopomer.

Incubation of monocytes with [^{2}H]oleic acid resulted in its incorporation into a variety of cellular lipids, but also in the appearance of a second deuterated fatty acid with a chromatographic retention time consistent with it being deuterated 16:1n-9, and with an *m/z* of its methyl ester derivative of 297.3, exactly the one predicted for such fatty acid (Figure 3D). Furthermore, analysis of the fragmentation spectrum of this second deuterated fatty acid showed major diagnostic peaks at *m/z* 62, 77, and 94 and minor ones at *m/z* 172, 220, and 264, very similar to deuterated oleic acid (cf. Figures 3C and 3D), suggesting a precursor/product relationship. Note that when labeling the cells with [^{2}H]oleic acid, only a second deuterated fatty acid is detected at significant quantities, i.e., [^{2}H]16:1n-9; we did not detect any other putative oxidation ([^{2}H]14:1n-9 or [^{2}H]12:1n-9) or elongation products ([^{2}H]20:1n-9). This indicates that 16:1n-9 is a bona fide stable fatty acid, not simply a mere intermediate in the oxidation of oleic acid.

To further establish the involvement of a β-oxidation reaction in the synthesis of 16:1n-9, we used etomoxir, an irreversible inhibitor of carnitine palmitoyltransferase-1 that, by preventing the formation of acyl carnitines, strongly inhibits mitochondrial β-oxidation (Lopaschuk et al., 1988). The monocytes were pretreated with or without etomoxir and then incubated with [^{2}H]oleic acid for 16 hr. In keeping with previous data, this procedure raised the cellular levels of [^{2}H]16:1n-9 by about 5-fold, and the presence of etomoxir reduced it by 40%–50% (Figure 3E). Moreover, when cells stimulated with AA were used, conversion of oleic acid to 16:1n-9 was further increased by 2-fold (0.75 ± 0.10 and 1.62 ± 0.25 nmol/mg protein of [^{2}H]16:1n-9 in resting and AA-treated cells, respectively; mean ± SE, n = 6), and this was equally inhibited by etomoxir. These results demonstrate that oleic acid is the cellular precursor of 16:1n-9 by β-oxidation, and that this process is accelerated in activated cells.

oxLDL Increases the Content of 16:1n-9 in Activated Monocytes

To assess whether the accumulation of 16:1n-9 could be regarded as a general feature of activated monocytes committing to a foamy phenotype and not a peculiarity of AA stimulation, we conducted experiments with oxidized low-density lipoprotein (oxLDL), a stimulus that also promotes lipid droplet formation in monocytes (Szilagyi et al., 2014). The cells were treated with 50 μg/ml oxLDL for 2 hr to allow direct comparison with AA. No significant changes were detected in phospholipid fatty acid levels after the different treatments (Figure 4A). In contrast, clear increases in TAG and CE levels were observed after oxLDL treatment, as well as in their constituent fatty acids (Figures 4B and 4C). Strikingly, 16:1n-9 was again, in relative terms, the fatty acid that showed the largest increase.

We also conducted studies with bacterial lipopolysaccharide (LPS; 100 ng/ml, 16 hr) (Figure 4, right column). LPS is a well-established proinflammatory stimulus which, unlike AA and oxLDL, fails to induce lipid droplet formation in human monocytes (our unpublished data). Fatty acid analyses revealed that, although the stimulus induced changes in the phospholipid fatty acid composition of the cells, most notably decreases of both AA and 22:6n-3 (Figure 4D), it did not induce detectable changes in the 16:1n-9 content of neutral lipids (Figures 4E and 4F). Collectively, these results show that stimuli that promote lipid droplet formation, i.e., AA and oxLDL, do substantially increase the level of 16:1n-9 in neutral lipids. In contrast, stimuli that do not induce lipid droplet formation, i.e., LPS, do not drive the cells to accumulate 16:1n-9 in neutral lipids. Cellular accumulation of this fatty acid may thus emerge as a molecular marker of lipid droplet formation in monocytes.

16:1n-9 Possesses Anti-inflammatory Properties

The unique distribution of 16:1n-9 among cellular lipids and the finding that its levels are increased during cellular activation suggest a specific biological role for this unusual fatty acid. To study this question, we prepared cells enriched in this fatty acid by incubating them with 10 μM 16:1n-9 for 2 hr in serum-free medium. This procedure results in the cells taking up the fatty acid and preferentially accumulating it in neutral lipids, in a manner similar to if they had been previously activated with stimuli inducing a foamy cell phenotype (Figure 5A). Figure 5B shows

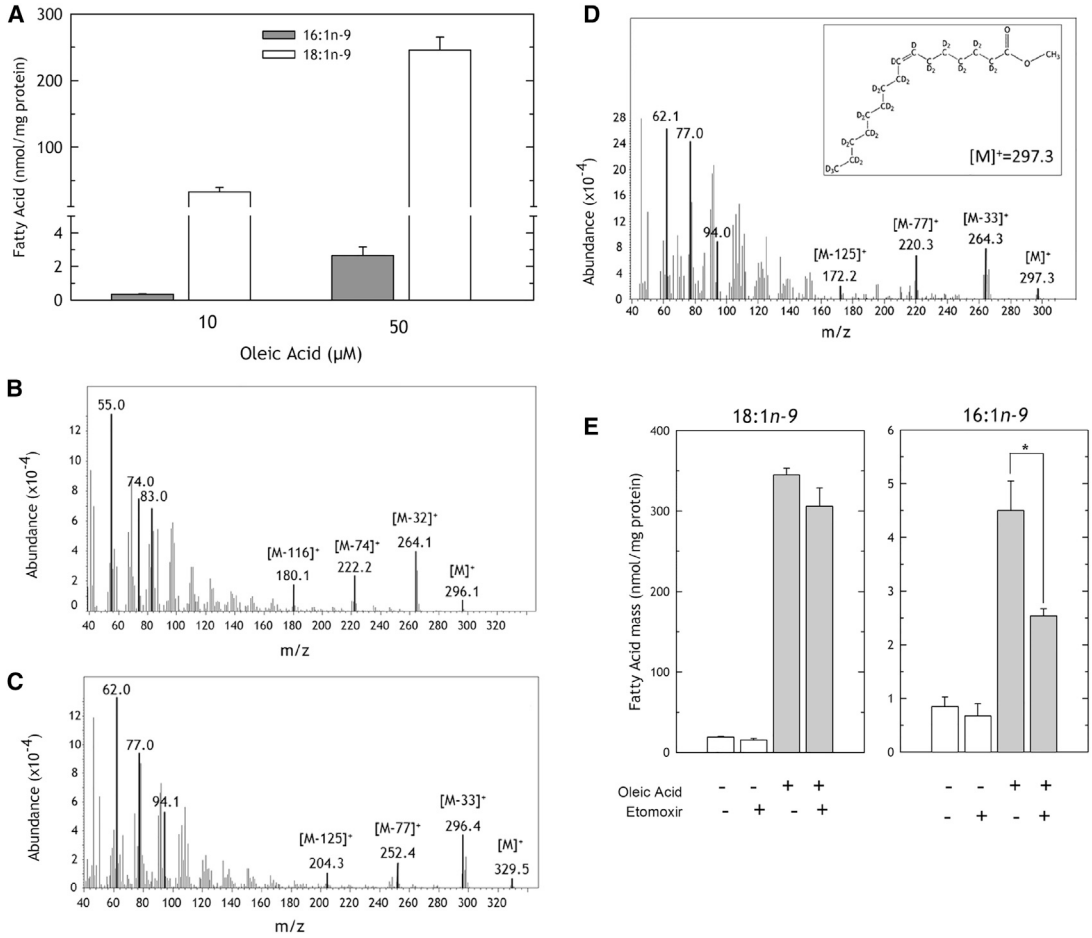

Figure 3. Metabolic Origin of 16:1n-9 Accumulating in Human Monocytes

(A) Enrichment of the cells with 18:1 results in a corresponding increase of the levels of 16:1n-9. The cells were treated with oleic acid at the indicated concentrations and, after 16 hr, the intracellular content of 16:1n-9 was analyzed by GC-MS. Data are shown as mean ± SE (n = 3).

(B) Fragmentation spectrum by electron impact MS of the FAME derivative of an 18:1n-9 standard.

(C) Fragmentation spectrum by electron impact MS of the FAME derivative of the uniformly deuterated isotopomer of an 18:1n-9 standard.

(D) Fragmentation spectrum of the FAME derivative of the uniformly deuterated isotopomer of 16:1n-9 from monocytes preincubated with deuterated 18:1n-9. The cells were incubated with 50 μM deuterated 18:1n-9 for 2 hr. Afterward the lipids were extracted and fatty acids were measured by GC-MS after conversion of the fatty acids to FAME derivatives. Inset shows the structure proposed for the methyl ester of deuterated 16:1n-9.

(E) Effect of etomoxir on the accumulation of 16:1n-9 in monocytes. The cells were incubated with 50 μM 18:1n-9 for 2 hr in the absence or presence of 30 μM etomoxir, as indicated. After lipid extraction, fatty acids were measured by GC-MS after conversion of the fatty acids to FAME derivatives. Data are shown as mean ± SE (n = 3). *Significantly different ($p<0.05$) from incubations with etomoxir.

that exposure of the 16:1n-9-enriched cells to LPS resulted in a markedly reduced expression of the *Il6* gene. Such a decrease was similar to that found when cells enriched in the omega-3 fatty acid 22:6n-3 were used. Of note, 16:1n-9 was significantly more potent than 16:1n-7, which exerted little effect (Figure 5B).

Despite the promising results, a study with human cells showed limitations such as the impossibility to conduct functional studies in vivo. To overcome these shortcomings we switched to mice, which allowed us to carry out studies on the effect of 16:1n-9 not only in isolated cells (peritoneal macrophages) but also at the whole-organism level. In preliminary experiments we sought to verify that macrophages from mice were comparable with human monocytes in terms of 16:1n-9 content and distribution, and thus effectively represented an adequate alternative. Strikingly similar to human monocytes, mouse macrophages were found to contain both 16:1n-7 and 16:1n-9 at comparable relative levels (three times as many 16:1n-9 as 16:1n-7 in both cell types) and, while 16:1n-7 resided almost exclusively in membrane phospholipids, 16:1n-9 was found in both phospholipids and neutral lipids (Figure S2A). When the distribution of 16:1n-9 was studied at a whole-organism level it was found that, of all tissues examined, only peritoneal macrophages contained more 16:1n-9 than 16:1n-7. In all other tissues, including serum, 16:1n-7 was consistently more abundant than 16:1n-9 (Figure S2B). The relative distribution of 16:1n-9 and 16:1n-7 among lipid classes (phospholipids, TAG, and CE) in the different tissues is shown in Figure S3.

Following the same procedure used with human monocytes, murine peritoneal macrophages were preincubated with 10 μM

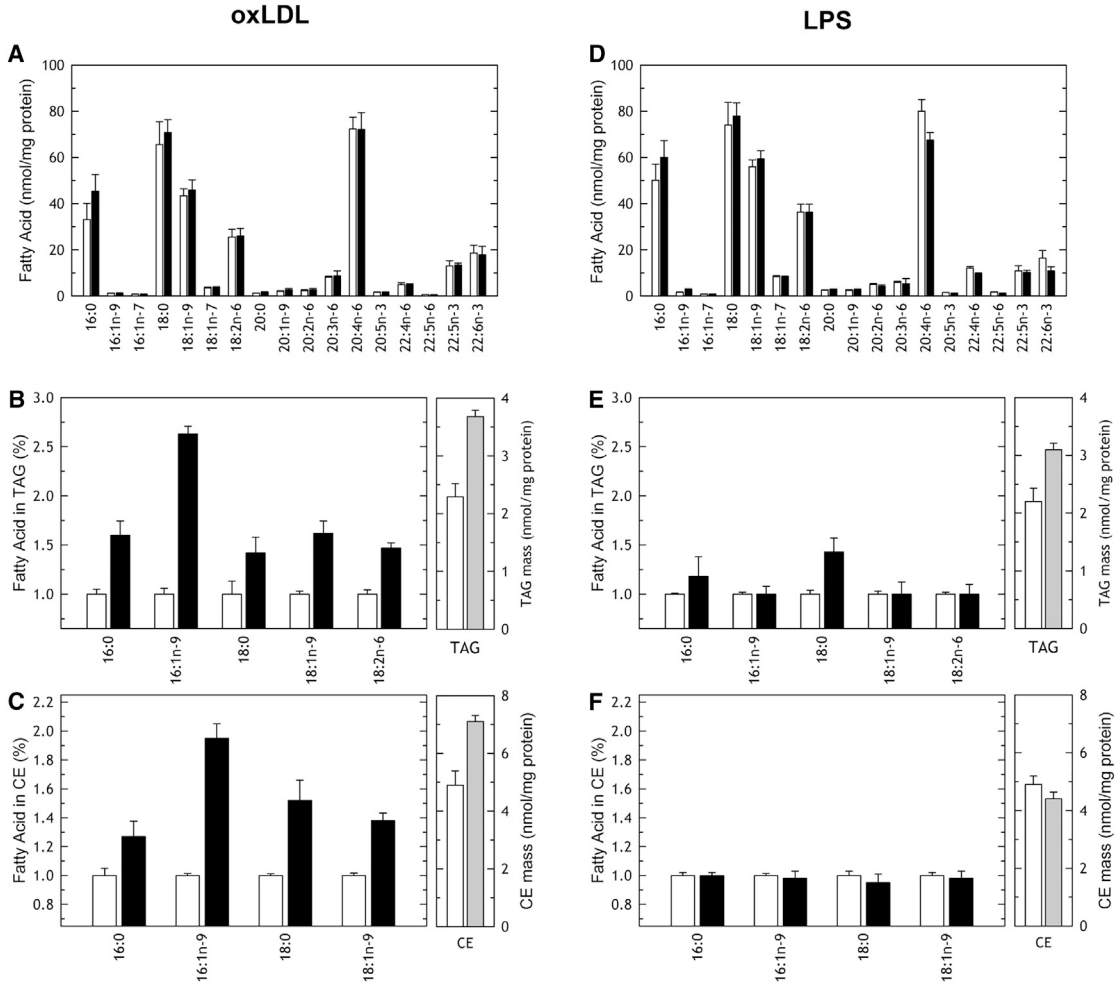

Figure 4. Stimulation of Monocytes with oxLDL but Not LPS Augments 16:1n-9 Levels in Neutral Lipids

(A–F) The cells were either untreated (open bars), or treated with 50 μg/ml oxLDL (closed bars in A–C) or 100 ng/ml LPS (closed bars in D–F) for 2 hr. After lipid extraction, fatty acids in phospholipid (A and D), TAG (B and E), or CE (C and F) were measured by GC-MS after conversion of the fatty acids to FAME derivatives. Data are shown as mean ± SE (n = 3).

16:1n-9 for 2 hr to obtain cells enriched in this fatty acid. 22:6n-3 and 16:1n-7 were also utilized for comparative purposes. The cells were stimulated with LPS and the effects on the expression of a number of proinflammatory genes investigated. As shown in Figure 5C, cells enriched in 16:1n-9 showed significant decreases in the expression of all genes tested, and such decreases were generally comparable with those found in the 22:6n-3-treated cells. When the cells were treated at once with both 16:1n-9 and 22:6n-3 (at maximal doses not compromising cell viability), the effects observed were no different from those observed with either fatty acid alone, suggesting that both fatty acids may share a common mechanism of action (Figure 5D). 16:1n-9 was significantly more potent than 16:1n-7 for all genes tested; 16:1n-7 had significant effects only in two of them, *Tnf* and *Nos2* (Figure 5C). These data show that 16:1n-9 has a spectrum of biological activity that is clearly distinguishable from that of 16:1n-7.

To assess the effects of 16:1n-9 at the whole-organism level, we carried out experiments in which the fatty acid was administered intraperitoneally to mice 1 hr before intraperitoneal injection of LPS for 6 hr. Afterward the animals were euthanized, peritoneal cells were harvested and cell samples matched by protein content, and the expression levels of *Il6* were studied. As shown in Figure 5E, both 16:1n-9 and 22:6n-3 inhibited *Il6* gene expression by the peritoneal cells isolated after the LPS challenge. Analysis of serum interleukin-6 (IL-6) protein confirmed a strong decrease in the amount of circulating IL-6 protein in the 16:1n-9-treated mice (Figure 5F). Unexpectedly, IL-6 protein levels in serum from 22:6n-3 treated cells were no different from those in serum from control untreated animals. The inability of 22:6n-3 to lower serum IL-6 levels has recently been reported in a study with human subjects (Muldoon et al., 2016), and may suggest that, unlike 16:1n-9-treated cells, the peritoneal cavity is not a major source for circulating IL-6 in 22:6n-3-treated cells; multiple sources for circulating IL-6 may exist in the 22:6n-3-enriched cells that compensate for the inhibition of *Il6* gene expression in the peritoneal cavity.

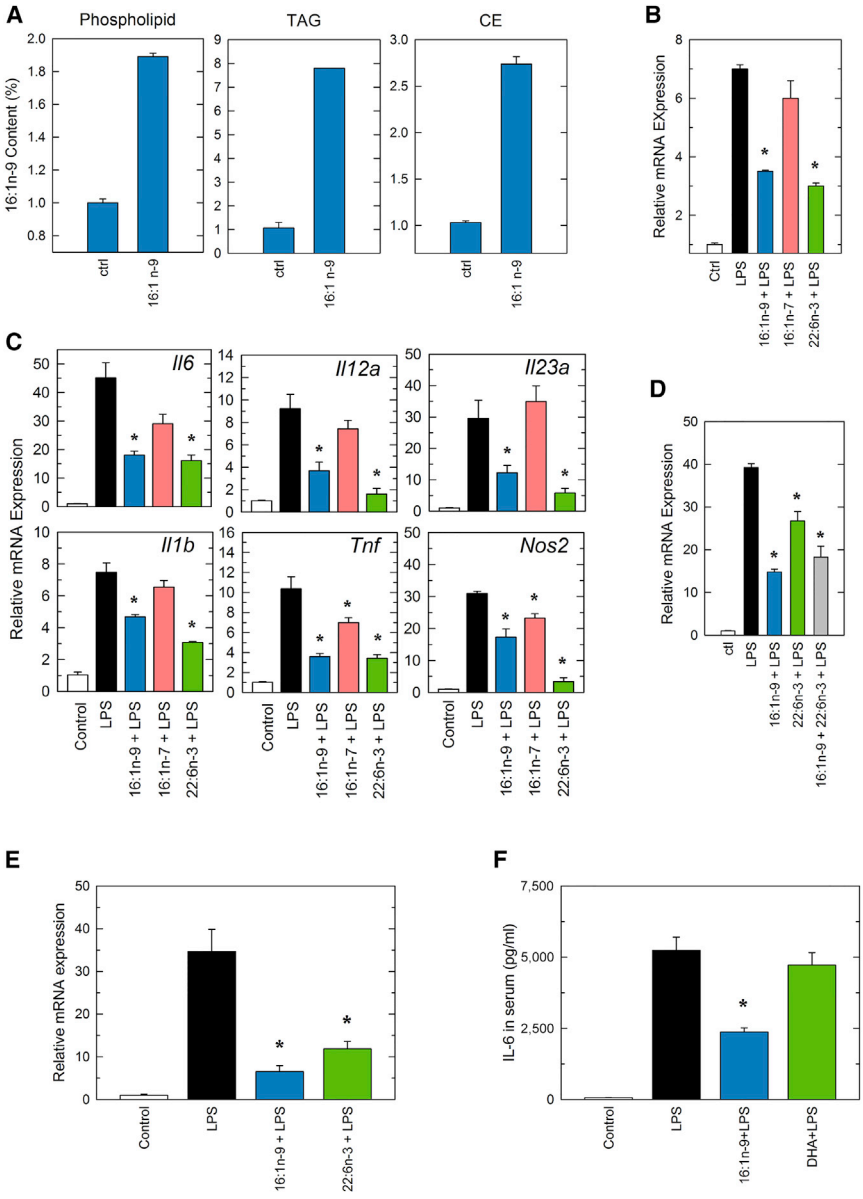

Figure 5. 16:1n-9 Possesses Anti-inflammatory Properties

(A) Human monocytes were preincubated with 10 μM 16:1n-9 for 2 hr and afterward the levels of this fatty acid in phospholipid, TAG, and CE were measured by GC-MS. Data are shown as mean ± SE (n = 3).

(B) Human monocytes, preincubated with 16:1n-9 (blue), 16:1n-7 (pink), 22:6n-3 (green), or neither (black), were stimulated by 100 ng/ml LPS for 6 hr, and *Il6* gene expression was measured. Data are shown as mean ± SE (n = 3).

(C) Murine peritoneal macrophages, preincubated with 16:1n-9 (blue), 16:1n-7 (pink), 22:6n-3 (green), or neither (black), were stimulated by 100 ng/ml LPS for 6 hr, and gene expression was measured. Results are shown as mean ± SE (n = 6).

(D) Murine macrophages, preincubated with 16:1n-9 (blue), 22:6n-3 (green), both (gray), or neither (black) were stimulated by 100 ng/ml LPS for 6 hr, and gene expression was measured. Results are shown as mean ± SE (n = 6).

(E) Mice were injected intraperitoneally with 20 mg/kg 16:1n-9 (blue), 22:6n-3 (green), or neither (black) for 1 hr. The mice were then injected intraperitoneally with 5 mg/kg LPS for 6 hr. Afterward the animals were euthanized, peritoneal cells were isolated, and *Il6* gene expression was analyzed. Results are shown as mean ± SE (n = 6).

(F) Serum from animals treated exactly as described in (E) was collected and IL-6 protein was assayed by ELISA. Results are shown as mean ± SE (n = 6). The fatty acids alone (i.e., in the absence of LPS stimulation) did not have any effect on the expression levels of the various parameters analyzed.

*$p < 0.05$, significantly different from incubations with LPS without fatty acids.

DISCUSSION

Monounsaturated fatty acids have long been recognized to be beneficial to the human diet (Schwingshackl and Hoffmann, 2012). Recently one of these fatty acids, 16:1n-7, was proposed as a novel anti-inflammatory mediator and positive correlate of insulin sensitivity in murine models of disease, albeit no conclusive evidence is yet available to support similar beneficial effects in humans (Hodson and Karpe, 2013; De Fabiani, 2011). A puzzling aspect of 16:1n-7 is that it may act differently depending on cell type, or even exert contradictory effects within a given tissue. While this diversity of actions and tissue-specific formation may underlie much of the contradictory findings observed (Hodson and Karpe, 2013; De Fabiani, 2011), the results presented here raise the provocative, hitherto unexplored possibility that isomers of 16:1n-7, being present at the same or neighboring locations, also possess defined biological activity that may overlap with and/or replace that of 16:1n-7. In other words, some effects previously attributed to 16:1n-7 may be due, at least in part, to positional isomers of 16:1n-7, and this study singularly points to 16:1n-9 (Figure 6).

Very little information is available on 16:1n-9. A PubMed search on 16:1n-9 returns only epidemiological papers where the fatty acid is merely listed as a minor constituent of certain tissues, with no regard for possible biological or biochemical implications (Zong et al., 2013; Wu et al., 2011; Ma et al., 2015; Lemaitre et al., 2010; Dai et al., 2015). Also, this fatty acid is barely mentioned, if at all, in the specialized lipid websites Cyberlipid Center (http://www.cyberlipid.org/), The Lipid Library (http://lipidlibrary.aocs.org/), and The Lipid Home (http://www.lipidhome.co.uk/). Because of this lack information and, given the relatively large increases of 16:1n-9 in activated cells, the prospect that 16:1n-9 could be envisioned as a candidate marker for foamy monocyte formation, a detailed characterization of the biochemical mechanisms of synthesis and cellular regulation of 16:1n-9 was warranted. In some of the studies mentioned above (Zong et al., 2013; Wu et al., 2011; Ma et al.,

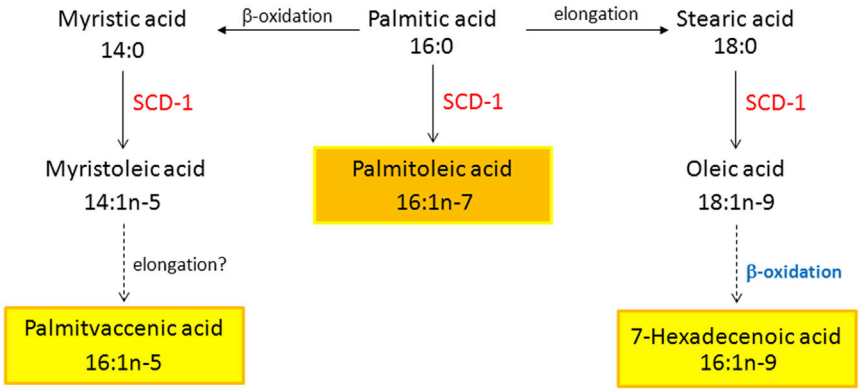

Figure 6. Palmitoleic Acid and Its Isomers in Mammalian Cells

Possible routes of biosynthesis are shown. Note that for all three isomers the double bond is introduced by stearoyl-coenzyme A desaturase (SCD) enzymes. Note as well, for the sake of completeness, that humans produce a fourth 16:1 isomer, called sapienic acid (*cis*-6-hexadecenoic acid, 16:1n-10). This isomer is produced by desaturation of palmitic acid at carbon 6 by a desaturase unrelated to SCD isoenzymes, and is expressed exclusively in sebum and epidermis, being absent in metabolically active tissues or immunoinflammatory cells. These characteristics make sapienic acid completely unrelated to the other 16:1 fatty acids.

2015) it was assumed but not demonstrated that 16:1n-9 derives from oleic acid; to the best of our knowledge, this work provides the first demonstration that this is actually so, and that it proceeds via mitochondrial β-oxidation.

It is remarkable that under proinflammatory stimulation conditions, the cells make neutral lipids and pack them into lipid droplets by using fatty acids that come from both the de novo biosynthetic route (16:0, 18:0, 18:1) (Guijas et al., 2012) and β-oxidation (16:1n-9, this study). While fatty acid biosynthesis and β-oxidation are not contradictory routes, i.e., they are not the reverse of one another as they occur at different subcellular locations and use distinct intermediates and cofactors, this work provides an example of a pathophysiological situation where the cells employ anabolic and catabolic routes at the same time to satisfy their metabolic needs. Moreover, the finding that 16:1n-9 derives from oleic acid, a major cellular fatty acid, does not imply that the former is merely produced as a consequence of an excess of the latter. Rather, production of 16:1n-9 appears to be tightly regulated by the activation state of the cell, likely because of its "biological value." It is noteworthy that no immediate β-oxidation products of other major fatty acids such as linoleic acid (18:2n-6) or AA (20:4n-6), are detectable, despite these fatty acids occurring at comparable or higher levels than oleic acid in human monocytes (Guijas et al., 2012) and mouse macrophages (Astudillo et al., 2011b). It has been reported that incubation of retinal rod outer segments with various fatty acids leads to the limited β-oxidation of only those whose products are used for N-acylation of retinal proteins (DeMar et al., 1996). Hence, it appears that limited β-oxidation of longer-chain unsaturated fatty acids may occur as a route to generate fatty acids with specific biological roles in cells. The unusual enrichment of phagocytic cells with 16:1n-9, not observed in any other tissue tested, is also compatible with specific biological role(s) for the fatty acid within the array of functions that these cells play in innate immunity and inflammation.

Lipid mediators are produced during inflammation in two temporal waves with opposite effects, when cells switch the type of mediators produced from pro- to anti-inflammatory (Dennis and Norris, 2015). This process is called "class switching" and is best exemplified by AA-derived eicosanoids, where the production of proinflammatory prostaglandins occurring immediately after the insult is progressively followed by accumulation of anti-inflammatory lipoxins, a process that initiates resolution of inflammation and the return to homeostasis (Dennis and Norris, 2015). Oxygenated derivatives of 22:6n-3 and other omega-3 fatty acids are also centrally involved in the resolution phase of inflammation (Serhan, 2014). Thus, cells appear to possess intrinsic mechanisms to dampen inflammation to avoid excessive damage that might lead to irreversible injury. Our studies provide a novel example of lipid class switching during inflammation whereby potent proinflammatory lipids such as free AA that are released by endothelial cells and other cell types present at various sites of injury may act on phagocytes to ultimately promote the synthesis and accumulation in these cells of a lipid with opposing effects on inflammation, 16:1n-9, which can later act to antagonize further proinflammatory insults.

Collectively, we believe that the current results identifying 16:1n-9 as a novel anti-inflammatory lipid point to many key areas of future research. We have presented evidence that at least part of its biological effects may be mediated by mechanisms similar to those triggered by 22:6n-3. As indicated above, 22:6n-3 and related long-chain omega-3 fatty acids (20:5n-3 and 22:5n-3) can be oxygenated by the same enzymes that oxygenate AA to generate biomolecules known as protectins, resolvins, and maresins (collectively called specialized pro-resolving mediators), which account for much of the biological activity of omega-3 fatty acids (Serhan, 2014). While hydroxy fatty acids can potentially be formed from any fatty acid, as long as it has at least one double bond, to our knowledge oxylipin derivatives of 16:1 fatty acids have not been described. However, based on a recent report describing the existence in mammalian cells of a novel family of lipids that result from the esterification of the hydroxyl group of a hydroxy fatty acid with another fatty acid (FAHFA) (Yore et al., 2014), it seems likely that these hydroxylated derivatives are produced at significant amounts in vivo. These novel lipid esters were found at carbons 5, 7, 9, 10, 11, and 12 of the hydroxy fatty acid, mostly hydroxystearic or hydroxypalmitic acids. While the biochemical route of formation of these hydroxy fatty acids was not ascertained, it is possible that at least some of them are derived from hydration of 16:1 fatty acids. Hydration of 16:1n-7 would yield 9- or 10-hydroxypalmitic acid, hydration of 16:1n-9 would yield 7- or 8-hydroxypalmitic acid, and hydration of palmitvaccenic acid (16:1n-5) would yield 11- or 12-hydroxypalmitic acid. Thus it is tempting to speculate on whether the anti-inflammatory effect of 16:1n-9 could be manifested, at least in part, after its conversion to a 16:1n-9-containing FAHFA molecule. If this was the case, exogenous 16:1n-9 could be envisioned to reproduce

the beneficial effects of FAHFA on insulin-stimulated glucose transport and overall metabolic improvement (Yore et al., 2014), opening new perspectives for the study of these novel anti-inflammatory lipids in metabolic disease.

SIGNIFICANCE

Results from this study describe the accumulation of an unusual fatty acid in the neutral lipid fraction of monocytes and macrophages, *cis*-7-hexadecenoic acid (16:1n-9), and provide evidence that such fatty acid possesses an anti-inflammatory activity that is significantly more potent than that of palmitoleic acid proper (16:1n-7) and in some cases comparable with that of omega-3 fatty acids. On the other hand, these results provide a starting point from which to explore the challenging possibility of whether detection of unusually high levels of 16:1n-9 in circulating monocytes could be regarded as a novel biomarker for the detection of foamy monocytes, and thus help to identify situations of early risk for atherosclerosis.

EXPERIMENTAL PROCEDURES

Cell Isolation and Culture Conditions

Human monocytes were isolated from buffy coats of healthy volunteer donors obtained from the Centro de Hemoterapia y Hemodonación de Castilla y León. Written informed consent was obtained from each donor. In brief, blood cells were diluted 1:1 with PBS, layered over a cushion of Ficoll-Paque, and centrifuged at 750 × *g* for 30 min. The mononuclear cellular layer was recovered and washed three times with PBS, resuspended in RPMI 1640 medium supplemented with 40 μg/ml gentamicin, and allowed to adhere in sterile dishes for 2 hr at 37°C in a humidified atmosphere of CO_2/air (1:19). Nonadherent cells were removed by washing extensively with PBS, and the remaining attached monocytes were used the following day. All procedures involving human samples were undertaken in accordance with the Spanish National Research Council Committee on Bioethics, under the guidelines established by the Spanish Ministry of Health and the European Union.

Murine resident peritoneal macrophages from Swiss mice (University of Valladolid Animal House, 10–12 weeks old) were obtained by peritoneal lavage using 5 ml of cold PBS, as described elsewhere (Diez et al., 1987). The cells were plated at 2 × 10^6 per well (six-well plates) in 2 ml of RPMI 1640 medium with 10% heat-inactivated serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, and allowed to adhere for 20 hr in a humidified atmosphere of 5% CO_2 at 37°C. Wells were then extensively washed with PBS to remove nonadherent cells. Adherent macrophages were then used for experimentation. When mice were treated with LPS, the animals were intraperitoneally injected with LPS at a dose of 5 mg/kg for 6 hr, after which the animals were euthanized with ketamine (100 mg/kg)/xylacine (10 mg/kg) and cervical dislocation (Meana et al., 2014). All the protocols and procedures were approved by the Institutional Animal Care and Usage Committee of the University of Valladolid, and are in accordance with the Spanish and European Union guidelines for the use of experimental animals.

GS-MS Analysis of Fatty Acids

The cells were washed twice with PBS, a cell extract corresponding to 10^7 cells was scraped in ice-cold water and sonicated in a tip homogenizer twice for 15 s and, before extraction and separation of lipid classes, internal standards were added. For total phospholipids, 10 nmol of 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine was added; for TAG, 10 nmol of 1,2,3-triheptadecanoylglycerol was added; and for CE, 30 nmol of cholesteryl tridecanoate was added. Total lipids were extracted and the lipids were separated by thin-layer chromatography, with n-hexane/diethyl ether/acetic acid (70:30:1 [v/v/v]) as the mobile phase. Spots corresponding to the various lipid classes were scraped and phospholipids were extracted from the silica with 800 μl of methanol followed by 800 μl chloroform/methanol (1:2 [v/v]) and 500 μl chloroform/methanol (2:1 [v/v]). TAG and CE were extracted with 1 ml of chloroform/methanol (1:1 [v/v]) followed by 1 ml of chloroform/methanol (2:1 [v/v]). Glycerolipids were transmethylated with 500 μl of 0.5 M KOH in methanol for 30 min at 37°C. 500 μl of 0.5 M HCl was added to neutralize. CEs were transmethylated as follows. Each fraction was resuspended in 400 μl of methyl propionate, and 600 μl of 0.84 M KOH in methanol was added for 1 hr at 37°C. Afterward, 50 μl and 1 ml of acetic acid and water, respectively, were added to neutralize. Extraction of FAMEs was carried out with 1 ml of n-hexane twice. DMOX derivatives of fatty acids were prepared starting from the FAMEs under mild conditions exactly as described by Svetashev (2011).

Analysis of FAME or DMOX derivatives was carried out in an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass-selective detector operated in electron impact mode (EI, 70 eV) equipped with an Agilent 7693 autosampler and an Agilent DB23 column (60 m length × 250 μm internal diameter × 0.15 μm film thickness) under the conditions described previously (Astudillo et al., 2011a; Guijas et al., 2012) with a slight modification of the procedure to improve separation of 16:1 FAMEs. In brief, oven temperature was held at 50°C for 1 min, then increased to 175°C at a rate of 25°C/min, then increased to 215°C at a rate of 1.25°C/min, with the final ramp being reached at 235°C at a rate of 10°C/min. Data analysis was carried out with the Agilent G1701EA MSD Productivity Chemstation software, revision E.02.00.

Real-Time PCR

Total RNA was extracted using Trizol reagent (Ambion). The cDNA templates were synthesized using M-MLW Reverse Transcriptase (Ambion) following the manufacturer's instructions. Quantitative real-time PCR analysis was performed in a LightCycler 480 (Roche) as previously described (Valdearcos et al., 2012).

Statistical Analysis

All experiments were carried out at least three times with incubations in duplicate or triplicate. Statistical analysis was carried out by Student's t test, with $p < 0.05$ taken as statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2016.04.012.

AUTHOR CONTRIBUTIONS

Conceptualization, C.G., M.A.B., and J.B.; Methodology, C.G., M.A.B., and J.B.; Investigation, C.G., C.M., and A.M.A.; Writing – Original Draft, J.B.; Writing – Review & Editing, C.G., C.M., A.M.A., M.A.B., and J.B.; Funding Acquisition, M.A.B. and J.B.

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