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Elaidate, an 18-Carbon *trans*-Monoenoic Fatty Acid, Inhibits β-Oxidation in Human Peripheral Blood Macrophages

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ABSTRACT

Consumption of *trans*-unsaturated fatty acids promotes atherosclerosis, but whether degradation of fats in macrophages is altered by *trans*-unsaturated fatty acids is unknown. We compared the metabolism of oleate (C18:1Δ9-10 *cis*; (*Z*)-octadec-9-enoate), elaidate (C18:Δ9-10 *trans*; (*E*)-octadec-9-enoate), and stearate (C18:0, octadecanoate) in adherent peripheral human macrophages. Metabolism was followed by measurement of acylcarnitines in cell supernatants by MS/MS, determination of cellular fatty acid content by GC/MS, and assessment of β-oxidation rates using radiolabeled fatty acids. Cells incubated for 44 h in 100 μM elaidate accumulated more unsaturated fatty acids, including both longer- and shorter-chain, and had reduced C18:0 relative to those incubated with oleate or stearate. Both C12:1 and C18:1 acylcarnitines accumulated in supernatants of macrophages exposed to *trans* fats. These results suggested β-oxidation inhibition one reaction proximal to the *trans* bond. Comparison of [1-^{14}C]oleate to [1-^{14}C]elaidate catabolism showed that elaidate completed the first round of fatty acid β-oxidation at rates comparable to oleate. Yet, in competitive β-oxidation assays with [9,10-^{3}H]oleate, tritium release rate decreased when unlabeled oleate was replaced by the same quantity of elaidate. These data show specific inhibition of monoenoic fat catabolism by elaidate that is not shared by other atherogenic fats. J. Cell. Biochem. 115: 62–70, 2014.

KEY WORDS: MACROPHAGE; *trans*-FATTY ACIDS; ELAIDIC ACID; FATTY ACID β-OXIDATION; ACYLCARNITINE; ENOYL-CoA δ-ISOMERASE

A 2009 World Health Organization study summarizing human clinical and observational studies of *trans* fatty acids concluded that these fats contribute significantly to cardiovascular risk [Kaushik et al., 2009]. Atherosclerotic disease is promoted by diets containing large amounts of long-chain saturated fat or by relatively small amounts of fat containing artificial *trans*-monounsaturates [Kummerow, 2009; Micha and Mozaffarian, 2009]. In either case, advanced atherosclerotic disease is characterized by vascular intima accumulation of semi-liquid cellular debris, consisting, in large part, of oxidized membrane phospholipids and cholesterol [Piotrowski et al., 1996; Craig et al., 1999]. The surrounding tissue is a mixture of proliferating smooth muscle, fibroblast-like cells, and "foam cells," a type of macrophage containing large amounts of lipid. In contrast, healthy vascular intima does not contain cell debris, proliferating cells, or lipid-laden macrophages. Under normal conditions, macrophages remove and recycle damaged cells to prevent the accumulation of toxic products. While it is known that macrophages in the atherosclerotic environment have altered metabolic and phenotypic features [Adamson and Leitinger, 2011], whether macrophages contribute to atherogenesis and, in particular, whether *trans*-unsaturated fatty acids have a direct role in these changes is unclear.

Studies of *trans*-fatty acid metabolism using whole animals and liver cells established their adverse effects on overall fat, cholesterol, and lipoprotein production. In Sprague–Dawley rats fed a diet with

Abbreviations: ACADL, long chain acyl-CoA dehydrogenase; ECI1, enoyl-CoA δ-isomerase 1; ECI2, enoyl-CoA δ-isomerase 2; PBMC, peripheral blood mononuclear cells.

Janelle R. Zacherl and Stephanie J. Mihalik contributed equally to this project.

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10% of energy as fat and 4% of the fat as elaidate (C18:Δ9-10 *trans*; (*E*)-octadec-9-enoate) for 8 weeks, visceral fat and liver lipid content increased compared to rats fed a 10% fat control diet (no *trans*-fat). This result led the investigators to speculate that the liver, muscle, and adipose tissue responses to *trans*-fatty acids are different from that of saturated fats with respect to its handling of glycogen, glucose, and other nutrients [Dorfman et al., 2009]. Other studies showed that *trans*-monounsaturated fatty acid feeding increased cholesterol and total triacylglycerol synthesis in rat liver, while HDL cholesterol concentration declined [Guzman et al., 1999]. Further, *trans*-fatty acids are incorporated into cell membranes to a degree quantitatively similar to their occurrence in the diet [Mensink and Katan, 1990; Sepulveda et al., 2010]. A meta-analysis of human clinical studies showed that *trans*-monounsaturated fatty acid ingestion increased cholesterol and total triacylglycerol synthesis, while HDL cholesterol concentration declined [Mozaffarian et al., 2009]. Although these metabolic consequences of *trans* fat consumption are consistent with known risk factors for cardiovascular disease, we hypothesized that chemically derived *trans*-fatty acids have unique biochemical effects for encouraging the accumulation of cellular debris in peripheral tissues, specifically in tissue macrophages.

Our purpose here is to identify whether high concentrations of lipids containing *trans* fats, such as may be found in semi-liquid atheromatous debris, are toxic via their effects on the tissue macrophages that normally remove and recycle dead cells. As a first step, we focused on how human macrophages degrade *trans* fats in β-oxidation. No previous work has specifically addressed the degradation of *trans* fats in humans, but studies in rats showed that the β-oxidation of elaidate, the major artificially generated *trans* fatty acid, was incomplete, resulting in the accumulation of 5-*trans*-tetradecenate in perfused rat hearts and liver mitochondria, respectively [Willebrands & van der Veen, 1966; Yu et al., 2004]. β-oxidation of fatty acids usually goes to completion without any specific intermediates accumulating [Liang et al., 2001; Wang et al., 2010]. Yu et al. [2004] proposed that this intermediate accumulation in rodents occurred because elaidate is a poor substrate for the acyl-CoA dehydrogenases involved in the early steps of β-oxidation (Fig. 1A). However, in humans, these same acyl-CoA dehydrogenases have very different substrate specificities and levels and patterns of expression [Chegary et al., 2009], suggesting that the catabolism of elaidate in humans may differ, with potentially important consequences, from that in rats. Furthermore, a poorly metabolized substrate entering β-oxidation can affect the flow of all substrates through the pathway and, thus, can affect both the quantity and quality of fatty acids available for signaling and membrane synthesis.

To test the hypothesis that human macrophages are defective in their ability to degrade *trans* fats, we derived fresh macrophages from peripheral blood monocytes [Robinson et al., 2009] and investigated how these cells process large quantities of elaidate, the major *trans*-C18-monounsaturated fatty acid, as compared to oleate, its *cis*-monounsaturated fatty acid isomer and to stearate, the 18-carbon saturated fatty acid (Fig. 1B). Additionally, after feeding the cells matched *cis*- or *trans*-octadecenoic acids or unprocessed and *trans*-processed dietary fat, we used MS/MS to assess fatty acid β-oxidation intermediates as acylcarnitines in cell supernatants. We determined fatty acid composition of cells after similar treatments using GC/MS.

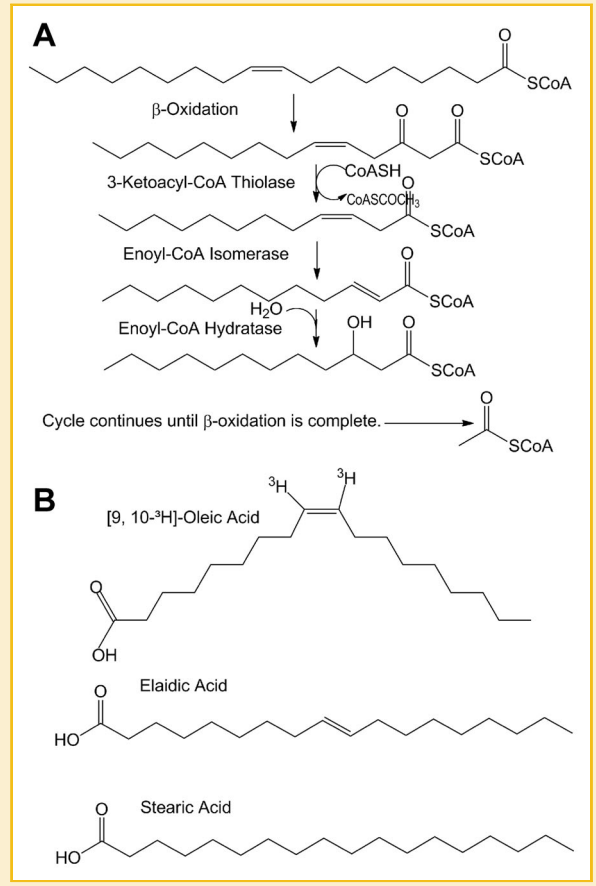

Fig. 1. Monounsaturated fatty acid β-oxidation cycle. A: Portion of the β-oxidation cycle specific for monounsaturated fatty acids where enoyl-CoA δ-isomerase moves the double bond from the 3-position to the 2-position. B: 18-Carbon fatty acids studied; IUPAC nomenclatures are oleate, (*Z*)-octadec-9-enoate; elaidate, (*E*)-octadec-9-enoate; stearate, octadecanoate.

Also, we compared the effect of accompanying *cis*- or *trans*-octadecenoic acids or stearate on tritiated water release from [9,10-^{3}H]oleate. We show that elaidate, *trans*-octadec-9-enoate, causes a previously uncharacterized disruption of monoenoic fat catabolism in primary human macrophages.

MATERIALS AND METHODS

CELL CULTURES

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats as described [Robinson et al., 2009] with the approval of The Institutional Review Board. The PBMCs were transferred to DMEM with 10% FBS, penicillin, streptomycin, 1 μg/ml carnitine (Sigma, St. Louis, MO), and 20 μg/ml human macrophage CSF-1 (Peprotech, Rocky Hill, NJ). For all experiments, 8×10^6 cells were plated on 10 cm^2 plates. After 2–3 days of incubation, the medium was replaced and all non-adherent cells were discarded. The PBMCs were used 4–7 days after plating. Hepatocytes (from human livers not suitable for transplantation), also obtained by protocols approved by the Institutional Review Board, were cultured in DMEM

supplemented with 10% FBS, insulin, gentamicin, amphotericin B, and dexamethasone (HMM SingleQuots, Lonza, Basel, Switzerland). Two to three days after plating, dexamethasone was removed from the cultures in two steps over 48 h. In all fatty acid treatment assays, 10% FBS was replaced with treatment fatty acid. Assays were performed 24 h after dexamethasone removal. Unless specified, media were obtained from Thermo-Fisher (Waltham, MA) and chemicals were from Sigma–Aldrich.

CELL PROTEIN DETERMINATION

Cell protein was quantified by bicinchoninic acid dye binding after hydrolysis of cells overnight at 4°C in 1 N NaOH followed by neutralization with HCl. For ^{14}C assays, proteins were determined using duplicate cell cultures.

ACYLCARNITINE ANALYSIS BY MS/MS

Macrophage cultures were incubated with fatty acids in DMEM with penicillin/streptomycin, 1 μg/ml carnitine, and 20 μg/ml of human CSF-1 for 7 days at 37°C in 5% CO_2. Fatty acids were from Grace Davison (Deerfield, IL) and were prepared as stock solutions of 3.5 mM fatty acids in 9% (1.4 mM) defatted albumin (BSA Fraction V, Sigma). They were added to DMEM at final concentrations of 100 μM in 400 μM albumin or as 400 μM albumin alone. For feeding with soy oil or partially hydrogenated soy oil, FBS was saturated with soy oil or partially hydrogenated soy oil containing 7% *trans*-octadecenoic acids. To saturate, FBS was incubated with 2 mg/ml of either oil for 20 min at 50°C, with vortexing, followed by filtration to remove fat not adsorbed to proteins. Cultures used 10% FBS saturated with soy oil or partially hydrogenated soy oil with untreated FBS as their controls. After the 7 days of fatty acid or fat feeding, 35 μl samples of cell supernatant were spotted onto Schleicher & Schuell Grade 903 filter paper, dried at room temperature, and stored at −20°C. Four wells of each treatment were assayed; each assay was performed using two 5 mm punches from the filter paper. Samples were processed for acylcarnitine MS/MS analysis essentially as described [Chace et al., 2001, Mihalik et al., 2010]. Briefly, punched paper with about 14 μl of dried tissue culture supernatant was reconstituted in 300 μl of methanol containing deuterated carnitine and acylcarnitines (free, C2-, C3-, C4, C5-, C8-, C14-, and C16-carnitine) as internal standards. Solvent was dried under nitrogen and then incubated in 3 N HCl in *n*-butanol at 65°C for 15 min to form butyl carnitine derivatives and dried again. Then samples were dissolved in 1:1 acetonitrile: 0.2% formic acid and injected into the electrospray ion source of the MS/MS (API Sciex 3000, Foster City, CA), scanning to record precursors with m/z 200–500 containing the butyl signature, m/z of 85.

FATTY ACID COMPOSITION OF CELLS BY GC/MS

Fatty acid incubation media were prepared as above (acylcarnitine analysis), using 100 μM final concentration of free fatty acids on BSA 1:4 M/M for 2 days. After the 2 days of fatty acid loading, cells were washed three times and harvested in 300 μl of PBS by scraping. Heptadecanoic acid (C17:0, 40 ng) was added as an internal standard. Lipids were extracted, de-esterified, and converted to methyl esters in a 3:1 methanol to methylene chloride solution with acetyl chloride catalyst at 75°C for 90 min. The resulting mixture was neutralized with 7% potassium carbonate, and esterified acids were extracted with hexane as described [Sepulveda et al., 2010], except that the samples were not derivatized with tetramethylsilane since the conversion to methyl esters was essentially quantitative [Sepulveda et al., 2010]. Extracts were dried under nitrogen and lipids were reconstituted in 50 μl of hexane; a 2 μl fraction was injected for separation by GC with identification of components by MS in a Hewlett–Packard 6890 instrument (Agilent Technologies, Santa Clara, CA). Chromatography was run in helium and used a 60 m nonpolar dimethylpolysiloxane column, 250 μm inside diameter, 0.02 μm film (DB-1MS, Agilent). The chromatography was started with 1 min at 50°C, followed by a 25°C/min ramp to 175°C. The column was held at 175°C for 10 min, followed by a 1°C/min ramp to 192°C, with a final increase at 10°C/min to 230°C. All results were normalized to the C17 internal standards.

COMPETITIVE TRITIUM RELEASE β-OXIDATION ASSAY

Tritium-release assays were performed after the method of Bennett [2007]. Specifically, PBMCs, prepared as described above in 10 cm^2 wells, were washed once in PBS and then incubated with 0.34 μCi [9,10-^3H]oleate ((*Z*)-octadec-9-enoate) (45.5 Ci/mMole; Perkin–Elmer, Waltham, MA) (Fig. 1) and 50 nmol fatty acids in 0.5 ml PBS with 1 μg/ml carnitine for 60 min at 37°C. The unlabeled fatty acids consisted of 25 nmol of oleate with 25 nmol of elaidate, stearate, or oleate. Fatty acid solutions were solubilized in α-cyclodextrin in PBS as described [Watkins et al., 1991]. For ^3H_2O collection, a column was prepared for each sample containing 750 μl of anion exchange resin in water (AG 1 × 8 acetate, 100–200 Mesh, BioRad, Richmond, CA). After incubation, the medium from each well was applied to the resin to bind the labeled fatty acids while the tritium released by β-oxidation of the fatty acids flowed through the column. The flow-through from the incubation and three 1 ml deionized water washes were collected and mixed with 10 ml of scintillation fluid (Ultima Gold, Sigma), followed by counting tritium released with a Packard Tri-CARB scintillation counter. Assays were performed in triplicate with triplicate blanks (incubation step omitted) for each sample. Standards contained a 500 μl aliquot of the incubation mix with 3 ml of deionized water and 10 ml of scintillation fluid. Hepatocytes were assayed identically except that the incubation time was reduced to 30 min. For experiments utilizing (+)-etomoxir sodium salt and antimycin A, the final quantities were 100 μM and 50 ng/sample, respectively.

^{14}C-WATER SOLUBLE PRODUCT β-OXIDATION ASSAY

Elaidate ((*E*)-octadec-9-enoate), [1-^{14}C], and oleate, [1-^{14}C], both 55 mCi/mMole, were from American Radiolabeled Chemicals (St. Louis, MO). For each incubation, 0.1 μCi of [1-^{14}C]fatty acid was mixed with the its free fatty acid to make 30 nmol total. Fatty acids were solubilized in α-cyclodextrin as above except that the final volume per 10 cm^2 well was 300 μl. Cells were incubated at 37°C for 60 min. Reactions were stopped by adding 120 μl of 18% perchloric acid to each well, followed by incubation for 15 min at 4°C. The soluble fraction was transferred next to glass tubes, and water-soluble reaction products were separated from the chloroform–methanol layer containing the lipids after Folch et al. [1957]. Water-soluble ^{14}C was then determined by scintillation counting.

MESSENGER RNA EXPRESSION STUDIES

The PBMCs were purified and plated as described above except that before plating, the final preparation was further purified by CD14 magnetic bead purification [Robinson et al., 2009] as described. Cells were incubated for 44 h in 30 μM fatty acids as above and, after washing, they were trypsinized and scraped into PBS and frozen at −80°C as a pellet. Messenger RNA was isolated using a RNeasy Mini kit (Qiagen, Valencia, CA), and first strand cDNA synthesis was performed by reverse transcription using random primers and Superscript III reverse transcriptase (Invitrogen, Grand Island, NY). Quantitative PCR was conducted using brilliant SYBR green fluorescent DNA intercalating master mix at 55°C for 40 cycles (Stratagene, La Jolla, CA) and the QuantiTect Primer Assays for human enoyl-CoA δ-isomerase 1 (ECI1) and enoyl-CoA δ-isomerase 2 (ECI2) (Qiagen). Substrate concentrations and incubation times were chosen to optimize for primary cell health and to investigate the substrate under a steady state.

RESULTS

To address whether, in human macrophages, *trans* fats are metabolized differently from naturally occurring dietary fats, we started with acylcarnitine profiling of media from cells cultured with fatty acids [Chace, 2001], a well-characterized method to detect blocks in the β-oxidation pathway (Fig. 1A). Lipid catabolic anomalies result in the accumulation of characteristic patterns of acylcarnitines. Specifically, in cultured cells, if inhibition occurs at any point of fatty acid catabolism, the cells will accumulate the reaction-specific acyl-CoAs. The cultured cells subsequently eliminate these excess acyl-CoAs by transesterifying them to form acylcarnitines that are transported out of the cell into the medium. When PBMCs were fed elaidate, they accumulated C12:1- and C18:1-carnitines in their media with $P < 0.01$ and $P < 0.05$ relative to oleate, respectively (Fig. 2A) with larger differences ($P < 0.01$ in all cases) relative to C12:1- and C18:1-carnitines for the albumin-only control or stearate-fed cells. The oleate-fed cells accumulated some of these same two species, C12:1- and C18:1-carnitines, relative to stearate fed cells ($P < 0.05$), but the effect of oleate feeding was quantitatively much less than the effect of elaidate feeding.

To determine whether these findings could be reproduced when dietary triglycerides, rather than the more toxic free fatty acids, were the source of the *trans*-unsaturated fatty acids, we compared acylcarnitine profiles of macrophages incubated for 1 week in 10% fetal bovine serum (FBS) to those incubated with FBS saturated with soy oil or with partially hydrogenated soy oil containing 7% elaidate with little residual other unsaturates (determined by GC/MS, see Methods Section) (Fig. 2B). In this experiment, the FBS saturated with soy oil caused a threefold increase in C12:1-carnitine relative to control FBS ($P < 0.05$), while FBS saturated with partially hydrogenated soy oil increased C12:1-carnitines eightfold relative to the control FBS ($P < 0.01$) and over twofold relative to the untreated soy oil ($P < 0.05$). The partially hydrogenated soy oil also increased C14:1-carnitine twofold relative to both of the other groups ($P < 0.01$). There was also a trend toward accumulation of C10:1-carnitine with the partially hydrogenated soy oil medium ($P = 0.07$) relative to the FBS alone.

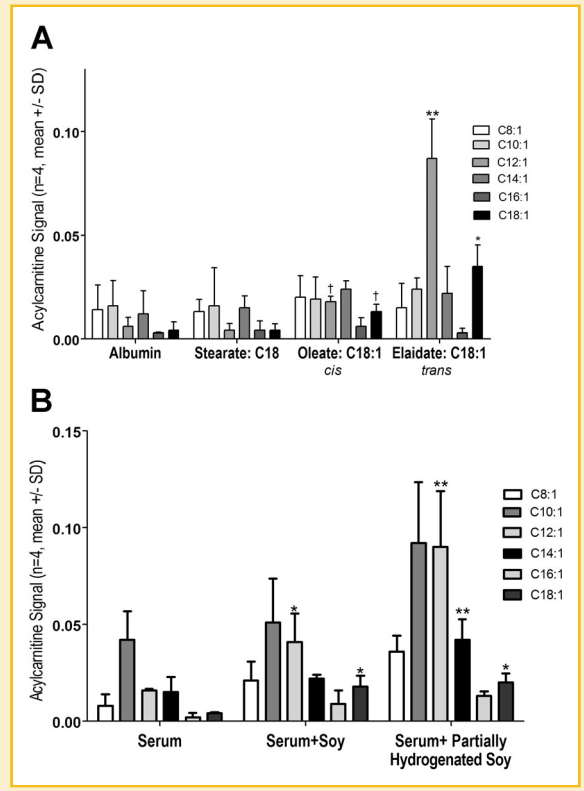

Fig. 2. Accumulation of acylcarnitines in supernatants after 44 h lipid incubation. Acylcarnitines accumulate in supernatants of human macrophage cultures fed unsaturated fatty acids. Determination by MS/MS, N = 4, mean ± SD is indicated. A: Effect of 100 μM fatty acids in albumin or albumin alone (left) on supernatant monounsaturated acylcarnitines at 44 h. $^{**}P < 0.01$ relative to all other conditions, $^{*}P < 0.05$ relative to oleate and $P < 0.01$ relative to albumin control or stearate feeding, $^{\dagger}P < 0.05$ relative to stearate-fed cells or albumin controls. B: Comparison of saturating 10% FBS with soy oil or with partially hydrogenated soy oil containing 7% *trans*-octadecenoic acids relative to untreated FBS. $^{*}P < 0.05$ relative to untreated FBS, $^{**}P < 0.05$ relative to soy oil. C10:1 groups serum versus serum with partially hydrogenated soy oil approached significance at $P = 0.07$.

Subsequently, we used GC/MS analysis to determine how treating macrophages with the different fatty acids on albumin affected total cellular fatty acid composition. Again, PBMCs were incubated for 44 h with 100 μM oleate, elaidic, or stearate, to assess steady state effects. Typical GC fatty acid separations are presented in Figure 3. When these data were compiled as percentile of each fatty acid for each treatment (Table I), quantifiable amounts of many more fatty acid species were found with elaidate rather than either oleate or stearate incubation. Unique species included chain-shortened products, such as both *cis* and *trans* C16:1, as well as the elongated fatty acids, C20:1, C20:2 and the polyunsaturated product C18:2. In addition, the proportion of C18:0 was greatly reduced in elaidate fed cells (by 80–90% relative to oleate or stearate, $P < 0.01$), with a lesser reduction of C16:0 (25–33%) relative to oleate feeding that did not reach significance. In the elaidate-fed macrophages, this saturated fraction was replaced by an increase in the total monounsaturated fraction, $P < 0.01$. Not surprisingly, the cells also accumulated large quantities of whatever fatty acid they had been fed.

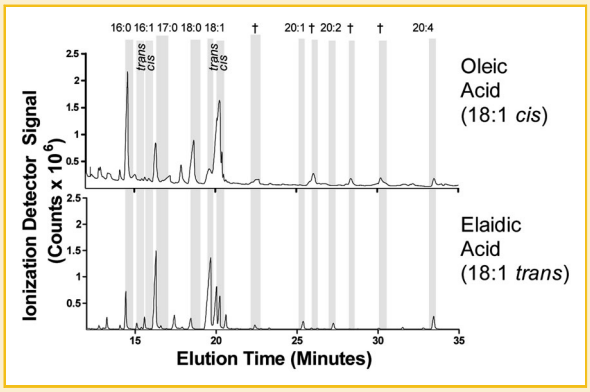

Fig. 3. Percent of fatty acid residues found in macrophages. Typical chromatograms of fatty acids isolated from whole macrophage cultures after feeding for 44 h with 100 μM fatty acids on albumin. The top chromatogram is from an oleate-fed culture, and the bottom is from an elaidate-fed culture. Key fatty acids identified by GC/MS are in shaded boxes labeled at the top. Quantitative data from several samples from all groups are presented in Table I.

The findings that with elaidate incubation, both *cis* and *trans* intermediates accumulate in the cells (Table I) and that a C12:1-carnitine intermediate accumulates (Fig. 2) are consistent with elaidate causing a delay at the enoyl-CoA δ-isomerization (ECI) step of the β-oxidation of C18:1 (ω-9) (Fig. 1). To test this more specifically, we utilized a tritium release assay using [9,10-^{3}H]oleate, a substrate that must pass through ECI, hypothetically the block point, before the tritium can be released. In addition, the experimental media included 50 μM unlabeled oleate with an additional 50 μM oleate, elaidate, or stearate. In macrophages, the added elaidate slowed the catabolism of [9,10-^{3}H]oleate by almost 70% relative to addition of oleate, while [9,10-^{3}H]oleate degradation increased when the accompanying fatty acid was stearate (Fig. 4A). When we performed the same experiment with isolated human hepatocytes (Fig. 4B), replacement of unlabeled oleate or elaidate with stearate again increased significantly the rate of oleate β-oxidation across the *cis*-double bond, as in macrophages. However, replacement with elaidate did not reduce the β-oxidation of oleate in hepatocytes.

The next studies (Fig. 4C) addressed whether the inhibition of β-oxidation by elaidate is specific to mitochondria or peroxisomes. The addition of the mitochondrial oxidative phosphorylation Complex 3 inhibitor Antimycin A [Zhang et al., 2012] reduced β-oxidation activity by 93%, 73%, and 88% with oleate, elaidate, and stearate competition, respectively. The CPT1 inhibitor etomoxir [Zhang et al., 2012] was slightly less potent, 83%, 77%, and 91%, respectively, in reducing β-oxidation activity. There was no significant difference in the quantity of residual β-oxidation activity when mitochondrial inhibitors were included.

To determine whether the effect of elaidate on oleate degradation in macrophages reflected a preferential initiation of β-oxidation on one isomer, we compared the β-oxidation rate of carbon 1 using [1-^{14}C]oleate and [1-^{14}C]elaidate (Fig. 4D). Here, we found that elaidate entry into the first round of β-oxidation was at least as rapid as that of oleate, with a trend toward increased rates $P = 0.16$, $n = 3$.

Finally, to address the discrepancy that oleate tritium release was inhibited by elaidate in PBMCs, but not in hepatocytes, we hypothesized that expression of ECI differs between the two cell types. Mitochondria express two ECIs, ECI1 and ECI2. Quantitative PCR (Fig. 5) showed that hepatocyte ECI2 mRNA was threefold more highly expressed relative to that in macrophages after oleate ($P < 0.05$) or elaidate ($P < 0.01$) incubation. Human hepatocytes to test the point further were not available.

DISCUSSION

Our study shows that in human macrophages, elaidate or *trans*-octadec-9-enoate, the major *trans*-unsaturated fat in artificially modified lipids, is poorly metabolized, resulting in its incomplete β-oxidation with C12:1-carnitine accumulation (Fig. 2). This intermediate forms where enoyl-CoA δ-isomerase (ECI) moves the double bond into a favorable position to complete unsaturated fatty acid β-oxidation (Fig. 1A). The tritium release studies in macrophages with labeled oleate verified that elaidate is a more potent inhibitor of

TABLE I. The Effect of Elaidate Feeding on Distribution of Fatty Acids in Macrophages

% of each species	Oleate (C18:1*cis*)	Elaidate (C18:1*trans*)	Stearate (C18:0)	Media only
C16:1 (*cis*)	1. 1 ± 2.2	2.2 ± 0.4	0	1.8 ± 1.6
C16:1 (*trans*)	0	1.5 ± 0.9	0	
C18:1 (*cis*)	45.2 ± 5.9	13.4 ± 7.9**	33.5 ± 9.7	27.6 ± 4.8
C18:1 (*trans*)	3.5 ± 4.2	49.2 ± 10.5**	7.9 ± 1.6	2.6 ± 4.5
C20:1		1.9 ± 1.3		
Total unsaturated fraction (%)	49.8	68.2**	41.4	32.0
C14:0	2.5 ± 0.6	1.8 ± 1.7	2.2 ± 3.0	2.2 ± 1.9
C16:0	26.2 ± 2.0	17.3 ± 9.7	23.3 ± 8.0	30.8 ± 3.7
C18:0	17.4 ± 2.0	3.6 ± 3.0**	29.2 ± 1.1**	24.3 ± 1.8
Ratio % C18:1(*cis*)/%C18:0	2.6	3.7	1.2	1.1
C18:2	0	3.2 ± 0.8	0	2.6 ± 3.8
C20:2	0	0.9 ± 1.1	0	
C20:4	3.2 ± 0.7	4.7 ± 1.2	3.8 ± 1.7	6.7 ± 6.7
C22:4	0.8 ± 0.6	0	0	

Methyl esters of fatty acids were prepared from cell lysates as indicated in Methods Section, separated by GC and identified by MS as shown in Figure 3. Cells were treated for 44 h with 100 μM fatty acids. Total fatty acids were normalized to 100%. Blanks indicate that average values are <1%. Significance of differences was determined only for products present at over 3% in one or more groups. A significant difference relative to both other groups, $P < 0.01$, is indicated ** $n = 3$ for oleate, elaidate, and media only and $n = 2$ for stearate. Mean ± SD is indicated.

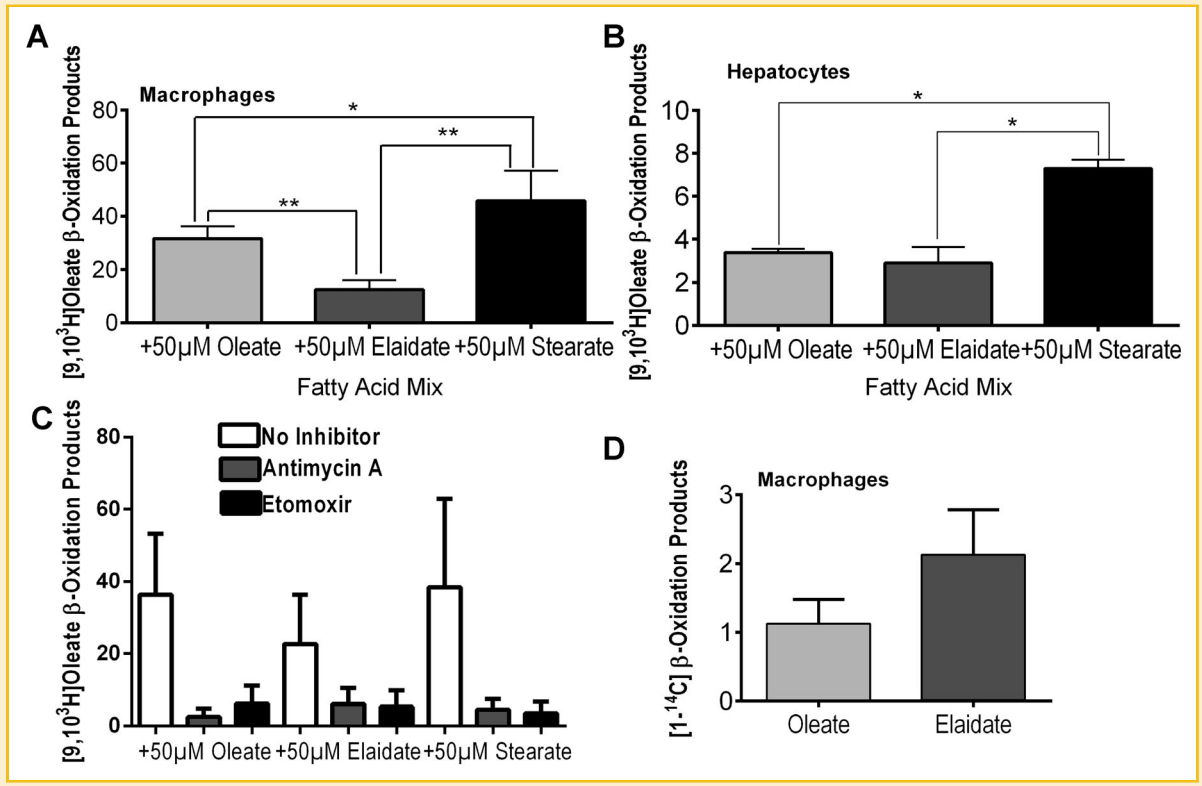

Fig. 4. Comparison of β-oxidation rates. In each case, cells were incubated in 100 μM total fatty acid and results are reported as nmol/mg protein/h. Means ± SD are shown. Matched cell cultures were used for all assays. A. In macrophages, tritium released from [9,10-^{3}H]oleate (see Fig. 1B) in 50 μM oleate with an additional 50 μM of oleate (left bar), elaidate (middle bar), or stearate (right bar) added as competitors, n = 4. Elaidate reduced the rate of oleate β-oxidation, $^{**}P < 0.01$. 50 μM Stearate increased the rate of oleate degradation, $^{*}P < 0.05$. B. An identical experiment using primary hepatocytes, n = 3. Stearate also increased the rate of oleate degradation, $^{**}P < 0.01$. C: Comparison of oleate β-oxidation rates when macrophages were treated with mitochondrial inhibitors. N = 5 for antimycin A (50 ng/sample) experiments, n = 3 for etomoxir (100 μM) using the same conditions as Figure 4A studies. D: Relative rate of the first round of β-oxidation for elaidate versus oleate in macrophages. Activity was measured using [1−^{14}C]-labeled fatty acids. $P = 0.16$, n = 4.

β-oxidation than an equal quantity of oleate (Fig. 4A). Furthermore, replacing half of the fat with stearate enhanced oleate β-oxidation, supporting the conclusion that the block occurs during the isomerization of the double bond (Fig. 4A,B). Finally, both *cis* and *trans* C16:1 accumulated in macrophages in response to elaidate feeding (Table I). We conclude that elaidate interferes with β-oxidation of all unsaturates at the isomerase step and this extra step may be rate limiting. Furthermore, inhibitor studies suggest that this block occurs in mitochondria.

Mitochondria contain two ECIs, ECI1, and ECI2. When mice null for ECI1 were fed a high oleate diet, *cis*-3-C12:1-carnitine accumulated [van Weeghel et al., 2012]. When ECI2 expression was reduced in ECI1 null fibroblasts, even more *cis*-3-C12:1-carnitine accumulated, verifying that C12:1-carnitine accumulates when ECI activity is blocked. Since our acylcarnitine analysis does not separate the *cis* and *trans* isomers, we could not identify the isomers. The high oleate-fed ECI1 null mice also accumulated small quantities of C18:1-carnitine [van Weeghel et al., 2012], as did our macrophages fed elaidate (Fig. 1), suggesting that even substrate entry into β-oxidation can back up if ECI activity is inhibited. Rat liver also contains two ECIs that will process the *cis* and *trans* products of the C18:1 fatty acids [Zhang et al., 2002], but this study is the first to show C12:1 accumulation from a β-oxidation limitation in primary human cells. We are also the first to show that in human cells excess elaidate can interfere with unsaturated fatty acid β-oxidation.

With elaidate as substrate, rat liver mitochondria also accumulated chain-shortened intermediates, but here C14:1-carnitine was identified rather than C12:1-carnitine [Yu et al., 2004]. The authors postulated that this product appeared because the long-chain acyl-CoA dehydrogenase (ACADL) had a low preference for the elaidate intermediate 5-*trans*-tetradecenoyl-CoA, relative to that for oleate intermediates. Similarly, the high oleate-fed ECI1 null mice accumulated some C14:1-carnitine, as well as C12:1-carnitine. Thus, it appears that in rodents β-oxidation of unsaturated fatty acids slows one round of β-oxidation prior to block identified in human macrophages. It is not surprising that the elaidate β-oxidation differs in humans, since null mouse models for the long chain acyl-CoA dehydrogenases do not fully recapitulate the human disorders [Chegary et al., 2009]. At least three mitochondrial acyl-CoA dehydrogenases are active in long chain fatty acid β-oxidation [Ensenauer et al., 2005], and a review comparing the two species has clearly shown that the mouse and human enzymes differ in both

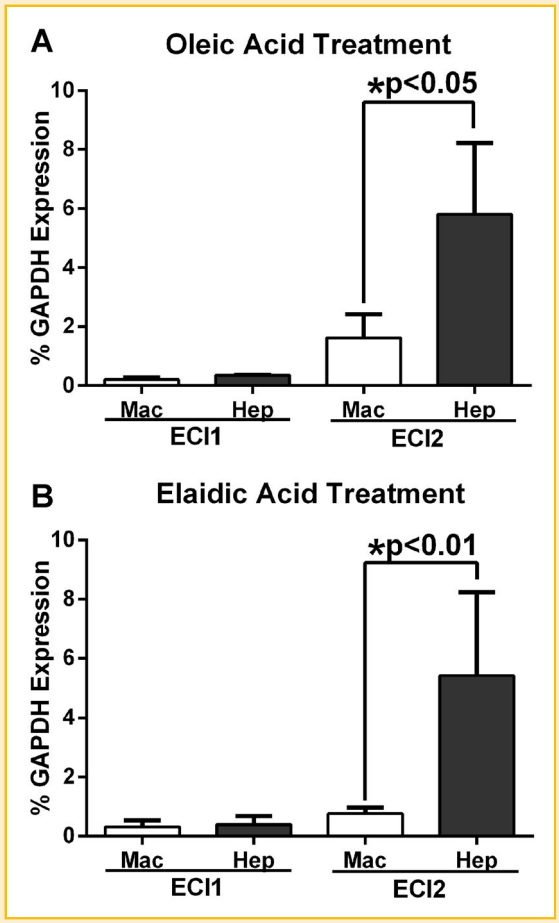

Fig. 5. Comparison of ECI1 and ECI2 expression by quantitative PCR. Cells ($n = 5$ for macrophages and $n = 2$ for hepatocytes) were treated for 44 h with 100 μM fatty acid as described. Fatty acid treatments did not affect expression significantly compared to each other. For ECI2, the expression differences in macrophages and hepatocytes reached significance of $P < 0.05$ for oleate treated and $P < 0.01$ for elaidate treated. For hepatocytes, range was used to test for significance instead of standard deviation.

substrate specificities and tissue distributions [Chegary et al., 2009]. In humans, C14:1-carnitine accumulates in very long chain acyl-CoA dehydrogenase deficiency [Chace, 2001].

However, the rodent studies and our human studies of elaidate β-oxidation share a central finding. Elaidate causes "leaky" β-oxidation, where intermediates accumulate as acylcarnitines, whereas β-oxidation normally occurs to completion [Liang et al., 2001]. Some acylcarnitine intermediate accumulation is also associated with adult Type 2 diabetes and obesity [Mihalik et al., 2010]. However, that accumulation is limited, involves several acylcarnitine species, and is thought to be associated with oxidative phosphorylation deficiencies. This single species C12:1-carnitine accumulation is consistent with a specific block in β-oxidation.

To support the hypothesis that the enzymatic block with elaidate is at the enoyl-CoA δ-isomerase (ECI) step, we used [9,10-^{3}H]oleate, a substrate that must pass through ECI before its label can be processed, to compare the effect of an equal quantity of elaidate to oleate on β-oxidation rates. We reasoned further that if we replaced half of the unlabeled oleate with stearate, the rate of oleate β-oxidation should increase relative to oleate alone. The results validated both these expectations. We also found, using [1-^{14}C]-labeled substrates, that elaidate goes through the first round of β-oxidation at least as rapidly as oleate, in agreement with an earlier study in hepatocytes [Guzman et al., 1999].

The same tritium competition studies using primary human hepatocytes (Fig. 4B) resulted in a similar increase in tritium release when stearate replaced half of the oleate but no corresponding alteration in β-oxidation rate when elaidate replaced it. Searching for possible explanations for these differences in heptocytes, we found that ECI2 was more highly expressed here than in macrophages (Fig. 5). Thus, macrophages are more susceptible than liver cells to the deleterious effects of *trans* fats, while liver may have inherently more capacity to mediate toxic fatty acids. Primary human hepatocytes for this work have been difficult to obtain, hence, the $n = 2$ for qPCR. Available hepatocytes were limited because those from fatty livers were excluded.

In experiments where we replaced the free fatty acids with mixed triglyceride-based human dietary fats, the C12:1-carnitine was still the primary intermediate when *trans* fats were used, reinforcing the original data and eliminating the possibility of an effect from the use of large quantities of elaidate in its free fatty acid form (Fig. 2B). Because partial hydrogenation produces a mixture of *trans* isomers at different carbons with elaidate still dominating, there was a less distinct pattern of accumulating intermediates. Curiously, controls fed serum saturated with regular soy oil also accumulated C12:1-carnitines, albeit to a much lesser extent than that caused by partially hydrogenated soy oil. Soy oil contains about 15% saturated fatty acids, 25% monounsaturates, and 60% polyunsaturates, with <1% *trans*-unsaturated fatty acids [List et al., 2005]. Apparently, large quantities of *cis*-polyunsaturated and monounsaturated fatty acids can challenge the capacity of the ECIs to degrade them, suggesting that in macrophages the isomerase step may be rate limiting for unsaturates. Consequently, these results show that triglycerides containing small quantities (7%) of *trans*-unsaturated fatty acids can still inhibit unsaturated β-oxidation.

To characterize further how macrophages accommodate the unnatural fat elaidate, we analyzed whole cell composition. While our acylcarnitine profiling suggested a β-oxidation block at C12:1, the shortest unsaturated product accumulating at detectable concentrations was C16:1. Both *cis* and *trans* products accumulated, again consistent with a rate limitation in the isomerization of the double bond in odd-chained unsaturates. This chain length discordance between whole cells and their excreted acylcarnitines was reminiscent of ACADL null mice, which accumulate C14:1-carnitine on a high fat diet [Zhang et al., 2007]. When these mice were fed labeled *cis*-C14:1(ω-9), it was elongated to C18:1 before incorporation into diglycerides. We found similarly that the elaidate-fed cells had a higher percentage of total C18:1 than the oleate-fed cells. However, these changes were balanced by a small reduction in C16:0 and an even greater reduction in C18:0. This alteration in C18:0 with an increase in C18:1 can also result from activation of stearoyl-CoA desaturase, an enzyme involved in enhancing membrane fluidity [Hodson & Fielding, 2012]. A similar reduction in saturated fatty acid concentration with a high *trans* fatty acid oral intake was reported in

a recent large in vivo human study [Kabagambe et al., 2012]. Overall, this altered fatty acid pattern in the elaidate-fed animals suggests that the cells may be responding by both elongating and desaturating the pool of elaidate fatty acid products prior to their incorporation into cells. An incidental finding in fatty acid-cultured PBMCs was a low percentage of C18:2 (Table I). Even the PBMC cultured in our basic 10% FBS media (Table I) had low C18:2 levels, suggesting that our incubation with large quantities of other fatty acids probably reduced the proportion of C18:2 to the limits of our detection.

The difficulties in processing elaidate by macrophages are important because *trans*-unsaturated fatty acids are incorporated into new cell membranes rapidly after they are consumed [Mensink and Katan, 1990]. Thus, when lesions occur in vascular intima after exposure to diets containing significant amounts of *trans* fat, macrophages would be faced with the extra challenge of degrading and removing cell membranes and related debris containing these poorly metabolized artificial fatty acids or their products. Removal of cellular debris is the central factor in progression of atherosclerosis, and macrophages are the primary responders in the prevention of this process. When lipid-rich debris cannot be removed rapidly, oxidized low density lipoprotein bound with phospholipids is hypothesized to contribute to the progression of lesions by overloading macrophages with lipid debris [Boullier et al., 2001; Zhu et al., 2005; Choi et al., 2008].

Overall, this study, which addresses the altered handling of *trans* fatty acids by macrophages, gives us a platform for investigating the metabolic effects of elaidic acid. We identified three specific effects of elaidate on human macrophage fatty acid metabolism. First, elaidate causes a specific block in β-oxidation of monounsaturates including oleate. This block is also associated with the accumulation of many unusual fatty acid intermediates. Finally, the fatty acid species in elaidate incubated cells differ from that of cells grown in normal medium or even from cells grown in medium highly enriched in oleate. Particularly notable is a reduction in C18:0 in elaidate-treated cells with a compensatory increase in unsaturated long-chain fatty acids (Table I). These lipid alterations will be useful in further characterization of the effects of *trans* fats on overall human macrophage physiology. We are particularly interested in the effects of accumulating products of elaidate metabolism on signaling pathways and on membrane function and composition. We recognize that *trans* fats may alter cellular properties by multiple pathways, including by increasing membrane rigidity. This alteration, in turn, affects cholesterol transport by macrophages [Fournier et al., 2012]. However, *trans* fats also inhibit β-oxidation with accumulation of unusual intermediates and changes in cell composition. Consequently, elaidate and its metabolites may affect cell metabolism and signaling in other unknown and unexpected ways.

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