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Journal of Cellular Biochemistry 116:524–532 (2015)

Journal of Cellular Biochemistry

Elaidate, an 18-Carbon Trans-monoenoic Fatty Acid, but Not Physiological Fatty Acids Increases Intracellular Zn^{2+} in Human Macrophages

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ABSTRACT

Artificial trans fatty acids promote atherosclerosis by blocking macrophage clearance of cell debris. Classical fatty-acid response mechanisms include TLR4-NF-κB activation, and Erk1/2 phosphorylation, but these may not indicate long-term mechanisms. Indeed, nuclear NF-κB was increased by 60 min treatment by 30 μM of the 18 carbon trans unsaturated fatty acid elaidic acid (elaidate), the physiological cis-unsaturated fatty acid oleic acid (oleate), and the 18 or 16 carbon saturated fatty acids stearic and palmitic acid (stearate or palmitate). However, except for stearate, effects on related pathways were minimal at 44 h. To determine longer term effects of trans fatty acids, we compared mRNA expression profiles of (trans) elaidate to (cis) oleate, 30 μM, at 44 h in human macrophages. We found that elaidate changed Zn^{2+}-homeostasis gene mRNAs markedly. This might be important because Zn^{2+} is a major regulator of macrophage activity. Messenger RNAs of seven Zn^{2+}-binding metallothioneins decreased 2–4-fold; the zinc importer SLC39A10 increased twofold, in elaidate relative to oleate-treated cells. Results were followed by quantitative PCR comparing cis, trans, and saturated fatty acid effects on Zn^{2+}-homeostasis gene mRNAs. This confirmed that elaidate uniquely *decreased* metallothionein expression and *increased* SLC39A10 at 44 h. Further, intracellular Zn^{2+} was measured using N-(carboxymethyl)-N-[2-[2-[2(carboxymethyl) amino]-5-(2,7,-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-phenoxy]-ethoxy]-4-methoxyphenyl]glycine, acetoxymethyl ester (FluoZin-3-AM). This showed that, at 44 h, only cells treated with elaidate had increased Zn^{2+}. The durable effect of elaidate on Zn^{2+} activation is a novel and specific effect of trans fatty acids on peripheral macrophage metabolism. J. Cell. Biochem. 116: 524–532, 2015.

KEY WORDS: TRANS FAT; INTRACELLULAR ZINC; ATHEROSCLEROSIS; MACROPHAGE METABOLISM

Small quantities of artificial trans-monounsaturated fat or large quantities of saturated fat promote atherosclerotic disease and disorders of energy metabolism [Kummerow, 2009; Micha and Mozaffarian, 2009]. Lipid metabolism is altered by trans-fatty acids; consequences include increased unsaturation of fatty acids in cell membranes [Sepulveda et al., 2010; Zacherl et al., 2014]. Fatty acids, especially trans- and saturated fatty acids, also mediate receptor-mediated cell signaling, which might in turn influence inflammatory cytokines that may modify many disease processes [Wymann et al., 2003; Hotamisligil, 2006; Wymann and Schneiter, 2008]. Because lipids have many metabolic and signaling effects, isolating cause and effect is difficult [Wymann and Schneiter, 2008]. In health, macrophages protect the vascular endothelium by removing cellular debris including cell membrane components, oxidized phospholipids and cholesterol, from the vascular intima. This should preclude accumulation of extracellular membrane debris, as well as related inflammation, cell proliferation, and atherosclerosis. Progressive atherosclerosis involves cycles of cell death and recruitment of

Grant sponsor: NIH; Grant number: R055208; Grant sponsor: NIH; Grant number: AR055208; Grant sponsor: Department of Veterans Affairs; Grant number: BX002490; Grant sponsor: NIH; Grant number: TR000146; Grant sponsor: NIH; Grant number: EB017268.

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Manuscript Received: 19 September 2014; Manuscript Accepted: 24 October 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 30 October 2014

DOI 10.1002/jcb.25002 • © 2014 Wiley Periodicals, Inc.

inflammatory cells by cytokines, with growth of atheromas until failure of vascular function occurs. How macrophage removal of cell membrane debris gets out of control is not fully understood, but it is exacerbated by saturated- and trans-monounsaturated fatty acids [Boullier et al., 2001; Choi et al., 2008].

We evaluated short- and long-term effects of fatty-acid on human macrophages. Classical response pathways were activated at ~60 min by all fatty acids at 30 μM, but only saturated fatty acids had extended (44 h) effects, particularly octadecanoic acid (stearate). Since trans fatty acids definitely affect continuing peripheral lipid metabolism, we followed these studies by exploratory 47,000-probe mRNA expression arrays. We compared stimulation of human macrophages at 44 h of exposure to 30 μM (E)-octadec-9-enoate, C18:Δ9–10 trans, commonly called elaidic acid (elaidate) or (Z)-octadec-9-enoate, C18:Δ9–10 trans, commonly called oleic acid (oleate). Effects on previously studied pathways were confirmed; elaidate partially blocks β-oxidation, with consequent effects on fatty acid unsaturation and other processes [Zacherl et al., 2014]. However, there was also reduced mRNA expression of zinc-binding metallothioneins and increased expression of a zinc importer in elaidate treated cells. This was an unexpected finding. Zinc regulates expression of inflammatory cytokines and innate immunity receptors in macrophages [Cousins et al., 2003; Haase and Rink, 2007]. The primary function of the metallothioneins is zinc storage. Zinc is essential to many biological processes, and interacts with thousands of proteins [Hambidge and Krebs, 2007]. Metallothionein-null mice survive, they cannot store zinc normally, and zinc supplementation is required for their survival [Tran et al., 1998; Davis and Cousins, 2000]. We confirmed the results of the array study by Quantitative PCR, and performed analysis of intracellular Zn^{2+}.

Our data indicate that effects of elaidate on cellular Zn^{2+} involve a durable increase in Zn^{2+} activity that cannot be attributed to fatty acid signaling by classical pathways. Large changes in transcription of lipid regulatory enzymes, including steroyl CoA desaturase, the ABCG1 fatty acid/cholesterol transporter, and hydroxymethylglutaryl CoA synthase, also occur selectively with long-term elaidate stimulation, suggesting that alternate intracellular metabolic regulation may lead to elevated Zn^{2+} and other pathological cellular responses.

MATERIALS AND METHODS

CELL CULTURE

Human peripheral blood monocytes were isolated from buffy coats as described [Robinson et al., 2009]. The cells were transferred to AIM-V medium containing 20 μg/ml of human CSF-1 (Peprotech, Rocky Hill, NJ). For quantitative PCR and expression array analysis, monocytes were CD14 purified with magnetic beads from Miltenyi Biotech (San Diego, CA) as described [Robinson et al., 2009]. Three days after isolation, CD14 purified cells or peripheral blood monocytes were treated with 30 μM oleate, elaidate, stearate, or palmitate, all carried by lipid-free bovine serum albumin (BSA) from Sigma–Aldrich, (St. Louis, MO), with 20 ng/ml human CSF-1 in Dulbecco's minimal essential medium (DMEM). Controls included a 10% FBS and a BSA only incubation. Fatty acid BSA preparation was as described [Watkins et al., 1991]. For the expression arrays, quantitative PCR and Zn^{2+} measurement, macrophages were treated for 15 or 44 h with fatty acids. For the NF-κB studies, macrophages were treated with fatty acids for 1 h.

NUCLEAR LOCALIZATION OF NUCLEAR FACTOR-κB

Human primary macrophages were cultured as for Zn^{2+} measurement. Cells were treated 1 h with or without the Zn^{2+} chelator 5 μM N,N,N′,N-Tetrakis (2-pyridylmethyl)ethylenediamine (TPEN) at 37 °C, with fatty acids and controls as indicated in results. Subsequent steps were performed at room temperature. Cultures were rinsed with PBS with 0.2 mM EDTA and fixed in 3% formaldehyde for 15 min, and rinsed again with PBS. Cells were blocked with 1% BSA for 30 min and then incubated with 500 μl of 1:250 NF-κB polyclonal rabbit antibody SAB4502610 Anti-NF-κB p65 antibody raised to a human P65 peptide sequence, in rabbit ~1 mg/ml, affinity isolated antibody, from Sigma–Aldrich. After rinsing, cultures were incubated with 1:1,000 Alexa Fluor 594 (red) donkey anti-rabbit for 1 h (Life Technologies, Grand Island, NY) and rinsed again. Cultures were also labeled in Hoechst fluor (Life Technologies) for one minute. Imaging used a Nikon TE2000 inverted phase-fluorescence microscope using a 14-bit 2000 × 2000 pixel monochrome CCD (Spot Instruments, Sterling Heights, MI). For red fluorescence, excitation was 536–556 nm with a 580 nm dichroic mirror and a 590 nm barrier. For blue fluorescence, excitation was 380–425 nm with a 430 nm dichroic filter and a 450 nm barrier. Fluorescent signal was photographed using 1.3 NA 40x or 100x oil objectives. Nuclei were defined by the blue Hoechst label to calculate the nuclear proportion of total cellular NF-κB label.

WESTERN BLOTS AND ELISA

Cells were lysed on ice in 150 mM NaCl, 50 mM Tris pH 7.5, 0.1% SDS, and 1% Triton-X-100, with phosphatase and proteinase inhibitors. Lysates were centrifuged to remove insoluble material. Protein concentrations were determined by Coomassie blue dye binding in acid (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA). Western Blots were essentially as described [Liu et al., 2012]; 16 μg aliquots of protein were run in sample buffer denatured by 10 min at 75 °C and separated on SDS–PAGE in pre-cast 4% – 12% gradient bis-tris-polyacrylamide gels (Life Technologies, Grand Island, NY). Proteins were transferred to polyvinylidine difluoride derivitized nylon membranes; 5% casein from bovine milk (blotting-grade blocker, Bio-Rad Laboratories) was used to block unreacted groups, followed by incubation with primary antibodies at 4 °C overnight. Blots were washed and incubated with horseradish peroxidase-labeled anti-rabbit IgG and anti-mouse IgG at 1:40,000 (Jackson Immuno Research, West Grove, PA). Proteins were detected by enhanced chemiluminescence (SuperSignal, ThermoScientific). Blots were re-probed for β-actin after the membrane was stripped for 20 min in 2% SDS in tris buffer with mercaptoethanol (Restore Plus Stripping Buffer, ThermoScientific). Mouse monoclonal anti-β-actin (clone AC-15, ascites fluid, Sigma) was used at 1:10,000. Phospho-p44/42 MAPK (Erk1/2) anti-Thr202/Tyr204 rabbit monoclonal antibodies, D13.14.4E (Cell Signaling, Danvers, MA) were used at 1:2,000; purified p44/42 MAPK (Erk1/2) rabbit polyclonal antibody was used at 1:1,000 (cell signaling). Rabbit polyclonal anti-

ZNF816A was used at 1:1,000 (Abcam, Cambridge, MA), to a synthetic peptide of N terminal amino acids 71–120 of Human ZNF816A. Mouse monoclonal anti-TLR4 (Clone# 285227) was used at 1 μg/ml (R&D Systems, Minneapolis, MN). Solid phase ELISA for TNFα used antibodies to recombinant human TNFα (Quantikine TNFα ELISA, R&D Systems).

cDNA ARRAY

Cells were CD14 magnetic bead purified [Robinson et al., 2009] and plated. After a 44 h incubation with 30 μM elaidate or oleate, media were removed and cells were washed twice in PBS. Macrophages were treated with 0.25% trypsin EDTA at 37 °C to loosen cells. Trypsin action was stopped with an equal volume of DMEM with 10% FBS. Cells were scraped from the plate and washed twice with PBS. Messenger RNA was extracted, reverse transcribed, and used to produce cDNA for analysis using the human U133 Plus 2.0 array (Affymetrix, Santa Clara, CA). This array contains the essentially genome-wide human genome U133 set and an additional 6,500 genes for analysis of over 47,000 human transcripts. The complete array probe-set and descriptions are available from the Affymetrix corporation on request.

QUANTITATIVE PCR

Peripheral blood monocytes were incubated for 15 or 44 h in 30 μM fatty acids, and quantitative PCR was performed as in our recent work [Zacherl et al., 2014]. In most cases QuantiTect (Qiagen) assays were used. These are pre-tested primer sets specified by GeneBank sequence, amplicon size, and approximate position. All extend across one or more introns. Products were verified by amplicon size on agarose gels (not shown). For human metallothionein-1X (MT1X) NM_005952 (468 bp), the QuantiTect amplicon extends across the first three exons and has an amplicon size of 157 bp. For human metallothionein-2A (MT2A) NM_005953 (466 bp), the QuantiTect amplicon extends across several mid-molecule exons and has an amplicon size of 139 bp. For human solute carrier family 39, member 10 (SLC39A10) NM_020342 (5,386 bp) the QuantiTect amplicon extended from exons 4–5 and is 138 bp. Results were normalized as a percentage of GAPDH expression, in this case using primers from NM_002046.3, F 5′-ACAGTCAGCCGCATCTTCTT, R 5′-GACAAGC-TTCCCGTTCTCAG, product 259 bp.

INTRACELLULAR Zn^{2+} ACTIVITY

Human primary macrophages were cultured on 35 mm MatTek (Ashland, MD) dishes containing 10 mm glass micro-wells. Cultures included the fatty acid treatments oleate, elaidate, stearate, and palmitate, 30 μM, and two controls, 10% FBS and BSA alone, in DMEM with 20 μg/ml human CSF-1. Cultures were incubated with all six combinations for 15 or 44 h. Cells were then rinsed with fat-free DMEM and 5 μl of 5 mM N-(carboxymethyl)-N-[2-[2-[2 (carboxymethyl)amino]-5-(2,7,-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl) phenoxy]ethoxy]-4-methoxyphenyl]glycine, acetoxymethyl ester (FluoZin-3-AM), Life Technologies, Grand Island, NY, was added in 1 ml of Dubelco's modified essential medium. After 30 min incubation, excess FlouZin-3 was removed by rinsing in fat-free media. Cells were photographed within 20 min in differential interference contrast and in green fluorescence to measure the Zn^{2+} activity, in fresh fat-free media using an AndorZyla VSC-00073 camera with 40x oil DIC H N2 optics; for fluorescence a 200 ms exposure and a mono 16 bit image was used. To quantify the intensity of signal, 11–21 images were measured per condition, with ~100 cells in each image.

RESULTS

NUCLEAR FACTOR-κB TRANSLOCATION

We hypothesized that the Zn^{2+} response to fatty acids might be associated with inflammation-related signaling, much of which operates through NF-κB activation. Since NF-κB translocation is rapid [Renard et al., 1997], 1 h fatty acids treatment was studied (Fig. 1). Elaidate, stearate, and palmitate-treated macrophages showed increased intranuclear NF-κB, $P < 0.0001$ versus controls (Fig. 1B). Oleate did not increase nuclear localization of NF-κB relative to controls. The increased intranuclear NF-κB in the trans- and saturated-fatty acid treated macrophages suggests common signaling at this short time point. As a control for specificity, adding the chelator, TPEN, 5 μM to the incubation media abolished differences in nuclear localization between groups (Fig. 1C). TPEN chelates Zn^{2+}, an established regulator of NF-κB activation. This is consistent with receptor activation of NF-κB translocation mediated by the fatty acids, at 1 h.

FATTY ACID RECEPTOR-MEDIATED RESPONSES AT 44 HOURS

Short-term cellular response to fatty acids is known to be mediated, in major part, by the innate immune system, particularly via toll-like receptor-4 (TLR4) [Cullberg et al., 2014]; the activation of NF-κB by elaidate, stearate, and palmitate, is consistent with this (Fig. 1). Whether durable activation of macrophages by fatty acids reflects longer-term effects on similar mechanisms was uncertain. To study this, effects of fatty acids on TLR4-related signaling mechanisms were determined at 44 h (Fig. 2). We included macrophages without added fatty acids, and non-activated lymphocytes, which have similar receptors, as additional controls. Quantity of TLR4 was reduced at 44 h by 30 μM fatty acids on albumin carriers, although TLR4 expression in stearate was much higher than in oleate, elaidate, or palmitate. Phospho-Erk1/2 was highest in controls and in stearate; total Erk1/2 was markedly reduced in the unsaturated fatty acids oleate and elaidate. These results indicate that the signaling pathway activity varies between the fatty acids at 44 h.

Since stearate has been shown to have distinct effects on TLR4 and phospho-Erk signaling compared to oleate, palmitate, and elaidate, we examined how the fatty acids affect long-term production of the important downstream inflammatory cytokine, TNFα, in cell supernatants at 44 h (Fig. 2F). This assay showed that stearate caused much greater production of TNFα at 44 h (Fig. 2F), a result highly significant relative to all other groups. This pattern matched greater phospho-Erk1/2 in stearate treated cells by densitometry (Fig. 2G), and might reflect maintenance of TLR4 in stearate treated cells (Fig. 2A). However, the ELISA for TNFα gave much clearer differences. We did additional blots for a nuclear transcription repressor, ZNF816 (Fig. 2C, right side). ZNF816 is widely expressed, including in macrophages and lymphoctyes, and has been associated

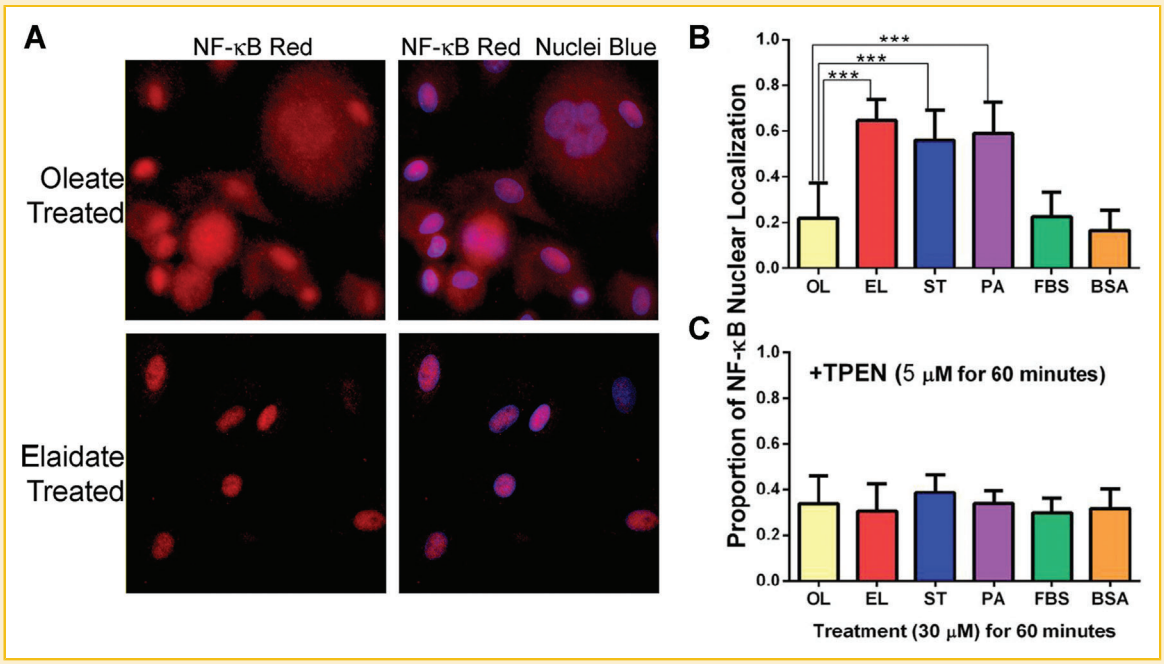

Fig. 1. Effects of fatty acid treatment on NF-κB nuclear localization. Macrophages were treated with 30 μM fatty acids for 1 h with and without 5 μM of the chelator TPEN. Immunofluorescence was conducted on the fixed cells and photographed. (A) Images of cells showing with low and high response. Oleate-treated cells had very little nuclear localization of NF-κB. Dark holes can be seen where the nuclei should be. In the elaidate-treated cells, the red NF-κB signal is largely co-localized with the blue nuclear label. (B) The proportion of NF-κB localized to the nucleus with each treatment at 1 hour. 30 μM elaidate-, stearate-, and palmitate- treated cells had significantly more nuclear localization than oleate- or control-treated cells; ***$P < 0.0001$. (C) The same experiment after the addition of 5 μM of the zinc chelator TPEN to the incubation medium. This treatment abolished differences, reflecting that components of the NF-κB activation cascade downstream of receptors require zinc for proper function.

with psoriasis [Sun et al., 2010]. Previous screening showed correlation of ZNF816 single nucleotide polymorphism isoforms with susceptibility to infection [Peck-Palmer et al., 2011], so ZNF816 might regulate macrophage inflammatory response. This transcription inhibitor was up-regulated by all fatty acid treatments at 44 h (Fig. 2C). However, a major fractional-size immunoreactive product seen in both untreated macrophages and lymphocytes was absent in fatty-acid treated cells (see Discussion).

GENOME-WIDE mRNA EXPRESSION IN ELAIDATE AND OLEATE TREATED MACROPHAGES

Since elaidate is strongly implicated in atherosclerosis, but did not show effects consistent with long-term receptor-mediated response that was seen in stearate (Fig. 2), we did genome-wide analysis of macrophage mRNA expression in elaidate versus oleate treated cells. Macrophages incubated for 44 h in 30 μM of the trans fatty acid, elaidate had decreased expression of six metallothionein genes relative to cells incubated with its natural cis isomer, oleate (Table I). In addition, the mRNA for the zinc transporter, SLC39A10 had enhanced expression. Other pathways where several mRNAs changed included in lipid metabolism; three key rate-limiting enzymes are also shown (Table I). Many other mRNAs changed significantly, including mRNAs for macrophage differentiation, immune function or stress response, and EGF family proteins (not shown). These pathways have precedents in macrophage differentiation studies. However, the zinc pathway was novel, and therefore it was studied further.

METALOTHIONEIN AND ZINC TRANSPORTER EXPRESSION IN FOUR FATTY ACIDS

We assayed MT1X, MT2A, and SLC39A10 mRNAs in CD14 purified macrophages after 44 h in fatty acids palmitate, stearate, oleate, or elaidate, a carrier control (bovine serum albumin, BSA), and a standard medium control including 10% fetal bovine serum (Fig. 3A). Results at 44 h were consistent with the expression array, and showed that the long-term effect on zinc regulating proteins were specific for elaidate. Elaidate-treated cells had the lowest expression for both metallothioneins, $P < 0.01$ compared to oleate; $P < 0.001$ compared to stearate and palmitate. The oleate-treated cells were intermediate in expression, $P < 0.01$ compared to all other treatments. The saturated fatty acid-treated cells, stearate and palmitate, had the highest metallothionein expression, $P < 0.001$ compared to all other treatments. Elaidate-treated cells showed the highest levels of SLC39A10 expression, $P < 0.05$ compared to palmitate, and $P < 0.01$ compared to oleate and stearate. A 15 h time point was also studied, and showed contrasting results (Fig. 3B). Changes in mRNAs from 15 to 44 h are indicated by the dashed lines between Figure 3A–B. At 15 h, expression metallothioneins in oleate- treated and controls was high compared to palmitate ($P < 0.05$), and to stearate and elaidate ($P < 0.01$). The expression of SLC39A10 was highest in controls, and lower but similar in all fatty acid treatments at 15 h. The zinc transporter expression varied significantly between fatty acids only at 44 h. Oleate and elaidate treatment caused decreases ($P < 0.001$, $P < 0.01$, respectively) in metallothionein expression from 15–44 h. In saturated fatty acids,

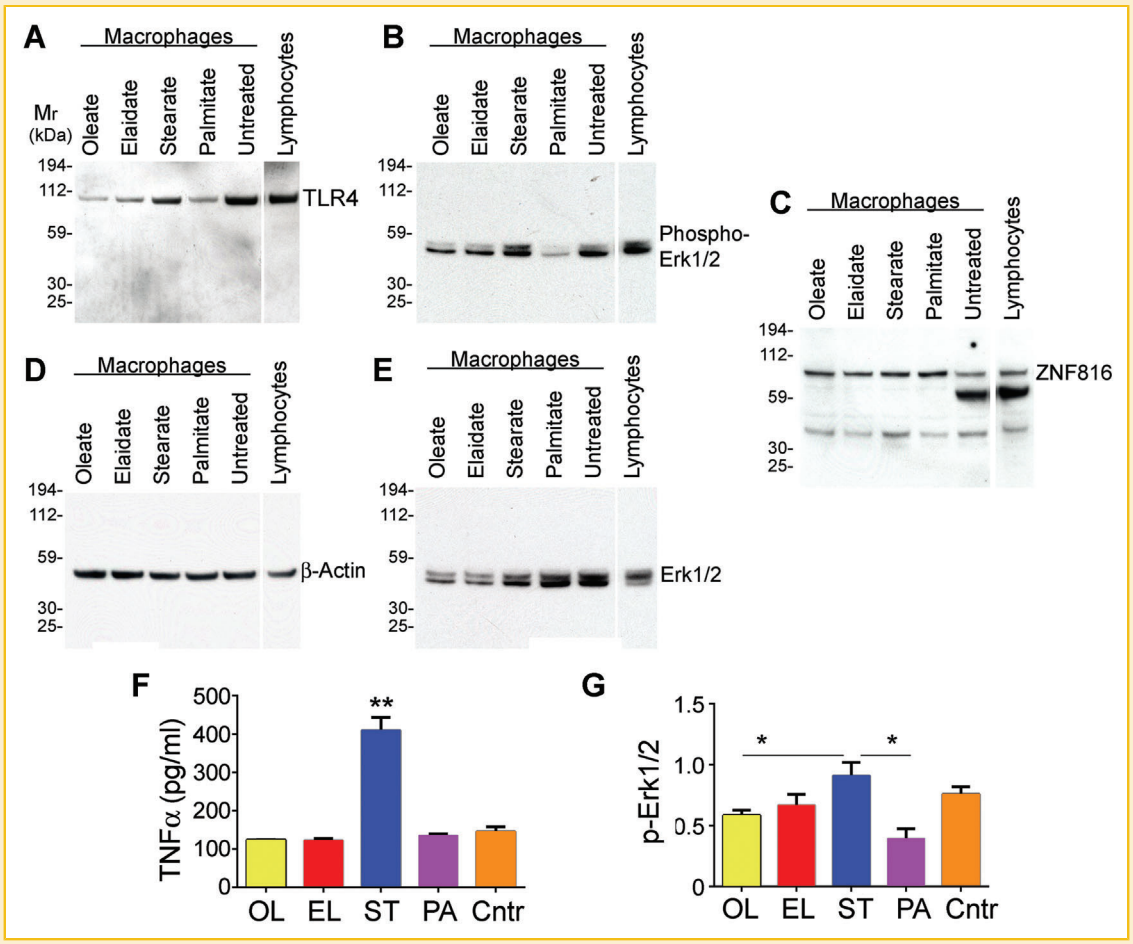

Fig. 2. TLR4, ZNF816, and Erk1/2 expression, Erk phosphorylation in 30 μM fatty acids and in control macrophages and lymphocytes, and TNFα production by ELISA all at 44 h. Westerns shown are single blots; irrelevant lanes between untreated macrophages and lymphocyte controls are deleted. Duplicate blots (not shown) for β-actin, phospho-Erk1/2, and Erk1/2 were used for densitometry. A. TLR4 expression in 30 μM fatty acids, 44 h. All fatty acids reduced TLR4 relative to untreated macrophages, but the loss of TLR4 in stearate was much less than with oleate, elaidate, or palmitate treatment. B. Phospho-Erk1/2 decreased with fatty acid treatment except in stearate. For densitometry, see (G). (C) An inflammation-related transcriptional repressor, ZNF816, was increased in fatty acid-treated macrophages. A lower MW form present in controls was lost in fatty acid-treated cells. (D) Actin controls for loading, used in densitometry. (E) Blots for total Erk1/2; compare with B. F. Effect of fatty acid treatment on TNFα expression in cell supernatants at 44 h, by ELISA. Stearate uniquely stimulates TNFα production at this time point. N = 4, mean ± range, $P < 0.01$ (G) Phospho-Erk1/2 relative to actin in two blots. Stearate is increased relative to all other conditions; differences are significant relative to oleate and palmitate, $P < 0.05$. N = 2, mean ± range.

stearate and palmitate, changes in metallothionein and SLC39A10 expression were minor and not significant. These results point, again, to differences in acute and chronic response to fatty acids in macrophages (see Discussion).

INTRACELLULAR Zn^{2+} ACTIVITY

Significant reductions in zinc-binding metallothioneins and increased expression of a zinc importer suggested that long-term response to elaidate might reflect chronic changes in intracellular Zn^{2+}. To determine how fatty acids affected Zn^{2+}, we used the fluorescent indicator FluoZin-3-AM (Fig. 4). Cells were treated with the membrane permeable acetoxymethyl ester of the dye, the zinc-binding fluor being released by cellular esterases. FluoZin-3 is quite specific for Zn^{2+} ions and is currently the most specific indicator for low concentrations of intracellular Zn^{2+}. At 44 h, the elaidate-treated cells had distinctly elevated Zn^{2+} activity, $P < 0.001$ compared to all other treatments, corresponding to the lowest expression of Zn^{2+} binding metallothioneins and elevated SLC39A10 (Table I, Fig. 3). None of the other fatty acid or control treatments had persistent low metallothionein expression or increased SLC39A10, and none had marked changes in Zn^{2+} at 44 h. At 15 h (Fig. 4B) elaidate, stearate, and palmitate had high and similar Zn^{2+}, but by 44 h, Zn^{2+} in saturated fatty acid-treated macrophages returned to control levels, while in elaidate-treated cells, Zn^{2+} increased from 15 to 44 h, $P < 0.05$. Intracellular Zn^{2+} did not vary in oleate-treated macrophages.

DISCUSSION

Saturated and trans-fatty acids promote atherosclerosis [Boullier et al., 2001; Zhu et al., 2005; Choi et al., 2008], but the mechanisms

TABLE I. Zinc Metabolism Genes With Highly Altered Expression and Selected Lipid Metabolism Genes

Zn-management genes	# Probes	OL	EL	log2 ratio	*P*-value
MT2A	1	6935	2317	−1.3	0.00002
MT1F	2	2753	847	−1.7	0.00002
MT1X	2	3839	1517	−1.6	0.00002
MT1G	2	4207	1618	−1.3	0.00002
MT1H	1	3566	1329	−1.3	0.00007
MT1E	2	2893	1035	−1.1	0.00003
SLC39A10	1	930	1739	0.8	0.00002
Key Lipid Metabolism Genes					
ABCG1	1	290	646	1.5	0.00002
SCD	2	1376	2447	0.8	0.00002
HMGCS1	1	2250	3963	0.8	0.00005

Gene, human gene name; Probes, number of separate probes are present on the array; if two, results are averaged.
Expression for oleate (OL) or Elaidate (EL) treated cells are averages of 20 measurements for each probe, using the Affymetrix human U133 Plus 2.0 array, containing over 47,000 DNA oligomers.
The log2 ratio indicates decreased (negative) or increased (positive) expression of elaidate relative to oleate.
The *P*-value is from a paired T test for difference between OL and EL.

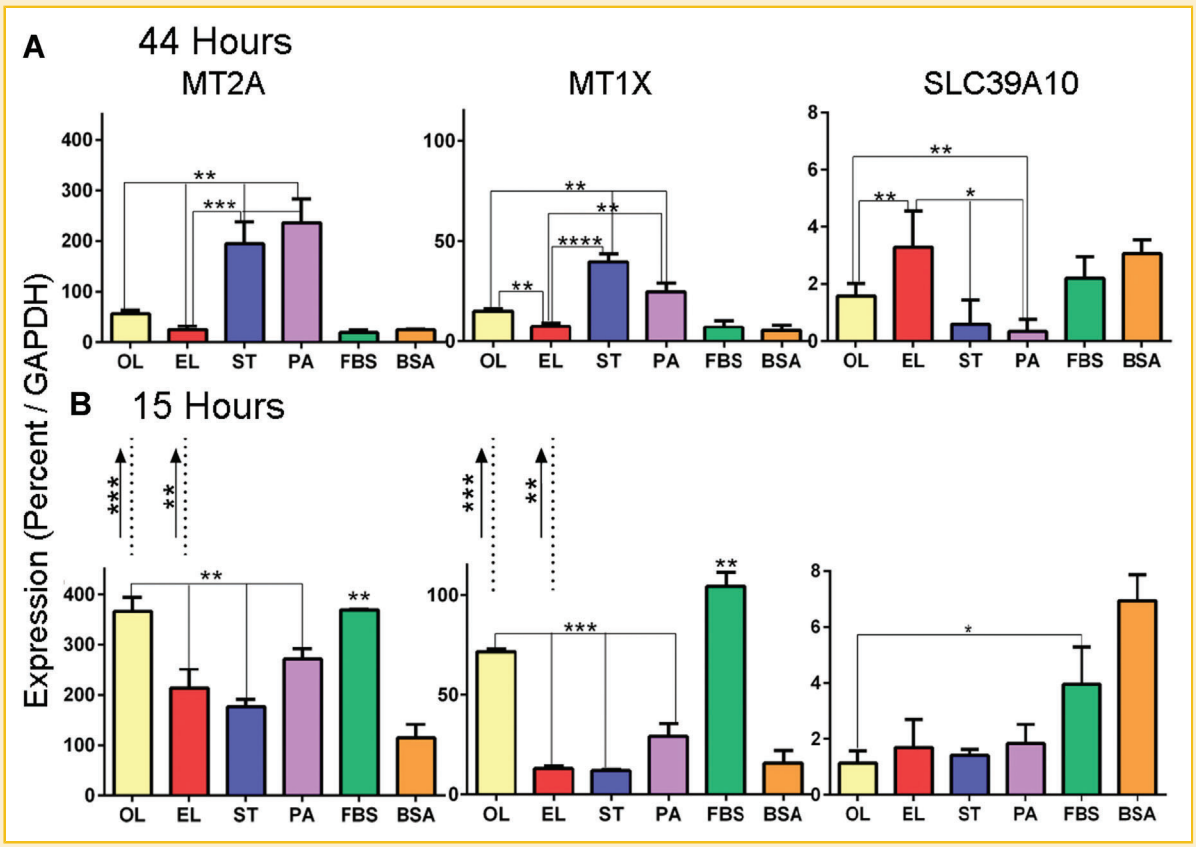

Fig. 3. Effects of fatty acid treatment on metallothioneins and on the zinc importer SLC39A10. Macrophages (n = 3–5) were treated for 15–44 h with 30 μM fatty acids on 0.1 mM BSA. Oleate (OL), elaidate (EL), stearate (ST), palmitate (PA), DMEM with 10% fetal bovine serum (FBS) and bovine serum albumin carrier without fatty acid (BSA). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. (A) Quantitative PCR as % GAPDH at 44 h. Elaidate had the lowest metallothionein expression and the highest SLC9A10 expression, significant at $P < 0.01$ or better relative to all other fatty acids. In contrast, the saturated fatty acids, stearate and palmitate, had highest metallothionein mRNAs and lowest SLC9A10 relative to elaidate, oleate, and the controls. (B) Quantitative PCR measurement of mRNA expression after 15 h. Metallothionein mRNAs were in elevated at this shorter time in oleate and complete medium with FBS. The zinc importer SLC39A10 was significantly higher in controls. Changes over time are indicated by the arrows between the graphs: Decreases in metallothionein mRNA in oleate and elaidate were the key changes with time.

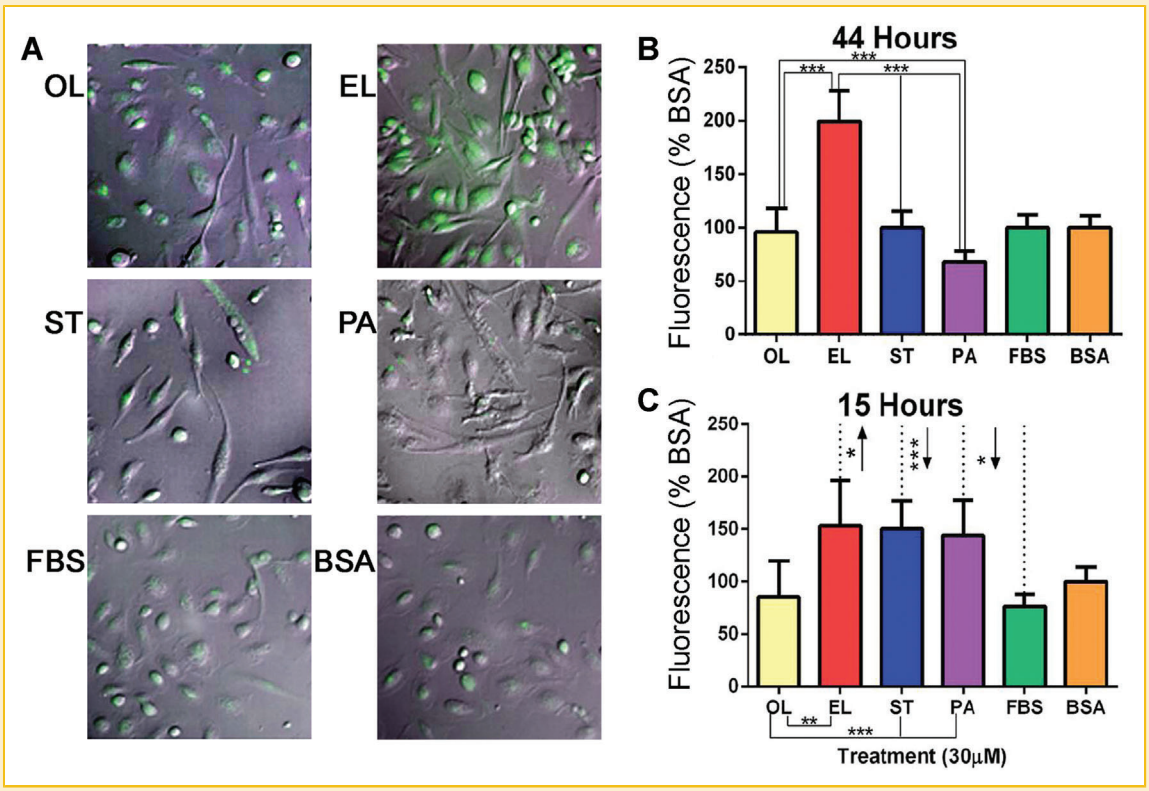

Fig. 4. Effects of fatty acid treatment on intracellular Zn^{2+} activity. Intracellular zinc activity after fatty acid incubation as measured with FluoZin-3. Macrophages were treated with 30 μM fatty acids for 44–15 h. FluoZin-3, AM was applied for 30 min. Cells were photographed in untreated media. (A) Images of cells at the 44 h time point. Note that the elaidate-treated cells had the most Zn^{2+} signal with palmitate-treated cells showing the least. (B) Quantified intracellular Zn^{2+} activity after treatment with fatty acid for 44 h. Elaidate-treated cells had the most labile zinc, while the palmitate-treated cells had the least. (*$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$) (C) Quantified intracellular Zn^{2+} activity after treatment with fatty acid for 15 h. Elaidate-, stearate-, and palmitate-treated cells had significantly higher labile zinc levels than the other three conditions (*$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$). From 15 to 44 h, Zn^{2+} activity increased only for EL-treated cells ($P < 0.01$), while it decreased or did not change significantly in all other treatments. Oleate or BSA did not significantly affect Zn^{2+} activity at 15–44 h.

causing this are not clear. We showed that saturated fatty acids promote macrophage activation over prolonged periods by classical mechanisms including TLR4 expression, phosphoErk1/2 activation, and TNFα production (Fig. 2). These findings fit with numerous other studies. However, response to the trans-fatty acid elaidate did *not* occur by similar mechanisms (Fig. 2). Our novel finding, by whole exome screening, was that gene products that affect zinc homeostasis are altered greatly by elaidate relative to oleate at 44 h (Table I). We validated these changes by PCR, with additional comparisons to saturated fatty acids (Fig. 3). Elaidate uniquely caused a sustained increase in Zn^{2+}, to at least 44 h (Fig. 4). This indicates a role for zinc signaling in trans-fat response, which previously was unknown.

Elaidate causes longer-term changes in cellular fat metabolism as well (Table I, lower part). This is not surprising since elaidate is difficult for macrophages to degrade. It stalls fatty acid metabolism and causes accumulation of intermediates of β-oxidation [Zacherl et al., 2014]. How these changes might be related to Zn^{2+}-handling gene expression (Fig. 3) is unknown, although chemicals that produce oxidative stress are known to upregulate metallothionein genes [Bauman et al., 1991]. The change in zinc-managing proteins at 44 h in elaidate is highly consistent and significant; no other fatty acids had parallel effects. The decreased metallothionein expression and increased intracellular Zn^{2+} suggest cause and effect, but further work will be needed to confirm this. Metallothioneins are a 10-isoform family with four major divisions [West et al., 1990]. These small proteins are largely localized to the Golgi apparatus in many cells including macrophages [Palmiter 1987]. Metallothioneins contain no aromatic side chains include ~30% cysteine, which enable the proteins to bind Zn^{2+} as well as heavy metals, nickel, copper, mercury, and silver [Palmiter, 1987].

The zinc transporter SLC39A10 was characterized in LLC-PK1 kidney proximal tubule cells [Kaler and Prasad, 2007]. It is a 40 kDa Zip family protein [Kambe et al., 2004], and increases the concentration of zinc in the cytosol in a saturable time and temperature sensitive manner [Kaler and Prasad, 2007]. The large increase in SLC39A10 in elaidate treated macrophages thus is consistent with a probable role in the long-term increase Zn^{2+} activity observed (Fig. 2B). There is probably also secondary regulation of SLC39A10; this is unstudied.

Studies of lipid activation of receptor-mediated pathways at 44 h (Fig. 2) show no detectable elaidate activity. Response of all of the fatty acids faded at 44 h, *except for stearate* where TLR4 protein,

phosphoErk1/2, and TNFα production all were maintained with significant differences relative to the other fatty acids. The selective effect of stearate on TNFα production and of elaidate on Zn^{2+} indicate strongly that saturated- and trans-fatty acids affect macrophage function by largely distinct mechanisms. We included blots of an inflammation-associated transcription repressor, ZNF816 [Sun et al., 2010; Peck-Palmer et al., 2011]. The high-molecular weight variant of this protein was uniformly increased in all fatty acid treatments, while fatty acid treatments eliminated a prominent lower MW band, of unknown functional significance, that is present in untreated macrophages or lymphocytes. This result is communicated solely to indicate that a long-term transcription suppression mechanism might regulate fatty acid response, while significant additional work will be needed to determine the significance of transcription repressor effects in long-term fatty acid pathology.

How elaidate downregulates metallothionein and upregulates SLC39A10 mRNAs is also unclear. It is unlikely, but possible, that this occurs through cell-surface fatty acid receptors (Fig. 2). Other possibilities include cellular regulation via lipid-related metabolic regulatory genes that change in elaidate, presumably secondary to the strong inhibition of β-oxidation by elaidate [Zacherl et al., 2014]. Three key regulatory enzymes that are significantly increased are shown in Table I. These include the ATP-binding cassette sub-family G-1, ABCG1, which mediates cholesterol and phospholipid transport, and regulates cellular lipid homeostasis, and is a strong candidate for regulation of inflammation and atherosclerosis [Westerterp et al., 2014]. Other regulatory genes in lipid related pathways whose expression doubled included steroyl CoA desaturase-1, SCD, and hydroxymethylglutaryl CoA synthase 1, HMGCS1. The protein encoded by SCD desaturates fatty acids, increasing membrane fluidity. The product of HMGCS1 is the statin target, the rate-limiting cholesterol synthesis enzyme. Both SCD and HMGCS1 are implicated in regulation of inflammation and in lipid-related metabolism and pathology [Liu et al., 2011; Rodriguez et al., 2012]. How these or other proteins might be linked atherosclerosis-inducing pathways is, as yet, uncertain.

Many inflammatory pathways are induced by fatty acids [Hotamisligil, 2006]. Toll-like receptor-4 is a key receptor responsible for innate immune response in atherosclerosis [Pasterkamp et al., 2004]. Recently, fatty acid signaling to toll-like receptors, particularly TLR4 in macrophage membranes, was shown to operate through fatty acids bound to the glycoprotein fetuin-A [Pal et al., 2012], increasing pro-inflammatory cytokines and oxidative stress. A common pathway stimulated by TLR4 is activation of nuclear factor-κB (NF-κB) through the IκB kinase (IKK) complex in macrophages [Karin and Ben-Neriah, 2000; Adamson and Leitinger 2011]. NF-κB is important in the induction of adhesion is cell proliferation surrounding atherosclerosis [De Martin et al., 2000). As short term effects, all of the fatty acids activate this pathway (Fig. 1), but in longer term assays, our results are consistent with TLR4 activation as a major pathway mediating stearate effects, but not effects of elaidate (Fig. 2).

In conclusion, our work suggests that the toxic trans fatty acid elaidate has long-term (44 h) effects driven, at least in major part, by tonically increased intracellular Zn^{2+}. Short-term NF-κB-related and intracellular Zn^{2+} signaling are induced by several fatty acids. However, only the 18-carbon saturated fatty acid stearate generated long term cytokine signals including TNFα, and it did not affect Zn^{2+} at 44 h. Thus stearate might mediate toxic inflammatory effects mainly, at longer times, by receptor mediated mechanisms. Overall, toxic fatty acid effects can initiate pathological function in macrophages by at least two largely distinct mechanisms: receptor mediated effects of fatty acids, and trans-fatty acid-mediated changes in cellular regulatory gene expression, including Zn^{2+} regulating proteins.

ACKNOWLEDGMENT

Opinions expressed are not those of the Department of Veterans Affairs.

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