CD36 Protein Is Involved in Store-operated Calcium Flux, Phospholipase A2 Activation, and Production of Prostaglandin E2*

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The scavenger receptor FAT/CD36 contributes to the inflammation associated with diabetes, atherosclerosis, thrombosis, and Alzheimer disease. Underlying mechanisms include CD36 promotion of oxidative stress and its signaling to stress kinases. Here we document an additional mechanism for the role of CD36 in inflammation. CD36 regulates membrane calcium influx in response to endoplasmic reticulum (ER) stress, release of arachidonic acid (AA) from cellular membranes by cytoplasmic phospholipase A2 (cPLA2) and contributes to the generation of proinflammatory eicosanoids. CHO cells stably expressing human CD36 released severalfold more AA and prostaglandin E2 (PGE2), a major product of AA metabolism by cyclooxygenases, in response to thapsigargin-induced ER stress as compared with control cells. Calcium influx after ER calcium release resulted in phosphorylation of cPLA2 and its translocation to membranes in a CD36-dependent manner. Peritoneal macrophages from CD36−/− mice exhibited diminished calcium transients and reduced AA release after thapsigargin or UTP treatment with decreased ERK1/2 and cPLA2 phosphorylation. However, PGE2 production was unexpectedly enhanced in CD36−/− macrophages, which probably resulted from a large induction of cyclooxygenase 2 mRNA and protein. The data demonstrate participation of CD36 in membrane calcium influx in response to ER stress or purinergic receptor stimulation resulting in AA liberation for PGE2 formation. Collectively, these results identify a mechanism contributing to the pleiotropic proinflammatory effects of CD36 and suggest that its targeted inhibition may reduce the acute inflammatory response.

Metabolic diseases, such as obesity, diabetes, and atherosclerosis, are associated with low grade inflammation that disrupts cellular homeostasis (1). Among the central mechanisms underlying this inflammation is compromised function of the endoplasmic reticulum (ER), which activates several cellular stress signaling pathways (1, 2). The scavenger receptor CD36 has been linked to the onset of ER stress and to some of its downstream sequelae. CD36 functions in cellular uptake of fatty acids (3, 4) and oxidized lipoproteins (5, 6), which under conditions of excessive uptake or abnormal metabolic processing can accumulate intracellularly to cause ER stress (2). In addition to facilitating lipid uptake, CD36 participates in the transduction of intracellular signaling events to activate stress kinases implicated in insulin resistance (7) and atherosclerosis (6). In ER-stressed macrophages, CD36 has been proposed to contribute to the suppression of ER survival pathways and the amplification of apoptotic cascades (2, 8). Studies in humans have suggested the importance of CD36 function in both lipid uptake and inflammatory processes (9). Increased CD36 expression on monocytes has been linked to monocyte activation in the diabetic state (10, 11), and the levels of a circulating CD36 form in plasma have been related to insulin resistance and atherosclerotic plaque instability (12, 13). Common CD36 genetic polymorphisms have been associated with risk of the metabolic syndrome (14), diabetes-linked coronary disease (15), and stroke (16) with variants that reduced CD36 protein expression associating with a protective atherogenic lipid profile (17). Elucidation of the cellular mechanisms that link CD36 to inflammation is central to the development of potential treatments for lipid-mediated inflammatory diseases.

A pathway that is central to inflammation in many tissues and closely integrates signaling events at the ER and plasma membrane (18) involves the release of arachidonic acid (AA) and its conversion to bioactive eicosanoids catalyzed by cyclooxygenases and lipoxygenases. Eicosanoids have pleiotropic effects in mediating acute inflammation (19–21) and have been implicated in diseases, such as atherosclerosis and thrombosis (22, 23), and in the endothelial dysfunction associated with diabetes (24). PGE2 is a major prostanooid metabolite of arachidonic acid, and its production by a variety of cells is part of the cellular response to stress (21).

The production of bioactive eicosanoids is tightly regulated because the overwhelming majority of cellular arachidonic acid is esterified to the sn-2 position of membrane phospholipids.

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2 The abbreviations used are: ER, endoplasmic reticulum; AA, arachidonic acid; BEL, bromoelon lactone (IE)-6-(bromomethylene)tetrahydro-3-(1-naphtalenyl)-2H-pyran-2-one); PM, plasma membrane; PYR, pyrrolidine (N-(2S, AR)-4-[biphenyl-2-ylmethyl]-isobutyl-amino)-1-12-(2,4-difluorobenzoyl)-benzoyl)-pyrrolidin-2-ylmethyl)-3-(4-(2,4-dioxothiazolidin-5-yldenemethyl)-phenyl)-acrylamide; SFK, Src family kinase; SSO, sulfo-N-succinimidyl oleate; PGE2, prostaglandin E2; cPLA2, cytoplasmic phospholipase A2α.
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The level of non-esterified AA is controlled by the relative activities of phospholipases, lysophospholipid acyl transferases (25), and arachidonic acid-specific CoA synthetases, such as ACSL4 (26). Cytosolic calcium-dependent phospholipase A₂α (cPLA₂α) plays a major role in AA release (18, 27) and is activated by increases in cytosolic calcium initially released from the ER and then sustained by calcium influx via membrane ion channels responsive to ER calcium depletion (28). The cPLA₂α enzyme is regulated through a series of phosphorylation events leading to its activation and translocation to the endoplasmic reticulum, where it selectively hydrolyzes phospholipids containing AA at the sn-2 position (29).

Membrane ion channels that replenish cellular calcium after ER calcium depletion are regulated by phosphorylation mediated by Src family kinases (SFKs) and Fyn kinase in particular (30–32), which is known to directly interact with CD36 (33, 34). The SFKs also regulate MAPKs (35–37), which include the cPLA₂s among their phosphorylation targets (38, 39). However, it is currently unknown whether CD36 plays a role in the regulation of AA release and eicosanoid production during cellular activation. The identification of such a role for CD36 would provide an important link between its pleiotropic functions and cellular inflammatory responses. Here, we demonstrate that CD36 participates in the activation of membrane calcium channels in response to ER calcium depletion or purinergic stimulation and in the phosphorylation and translocation of cPLA₂α to membranes to release AA from phospholipids.

EXPERIMENTAL PROCEDURES

Materials—Fura-2/AM and pyrrolidine (PYR) were purchased from Calbiochem, and racemic bromoenoil lactone (BEL) was purchased from Cayman Chemical. Thapsigargin and Fluo-4/AM were from Invitrogen. Antibodies for CD36 were from Abcam (Cambridge, MA) or Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); antibodies for β-actin, phospho-cPLA₂α, cSrc, Yes, Fyn, and Lyn were from Santa Cruz Biotechnology, Inc.; antibodies for phospho-ERK1/2, ERK1/2, and COX-1 were from Cell Signaling (Danvers, MA); Na⁺/K⁺-ATPase α6F was from the Development Studies Hybridoma Bank (University of Iowa); calnexin was from Stressgen (ENZO Life Sciences); and COX-2 and caveolin-1 were from BD Biosciences. Fatty acid-free bovine serum albumin fraction IV and other chemicals were from Sigma-Aldrich. Sulfo-N-succinimidyl oleate (SSO) was synthesized as described previously (40), and purity was determined to be greater than 95% by thin layer chromatography.

Cells and Culture—Chinese hamster ovary (CHO) cells were maintained in Ham's F-12 medium containing 10% fetal bovine serum, 200 units/ml penicillin, and 50 μg/ml streptomycin. CHO cells stably expressing wild type and mutated CD36 where C-terminal lysines (Lys⁴⁶⁹ and Lys⁷³⁷) were substituted by alanine (K/A CD36) were generated as described previously (41). Transfection of CHO cells was accomplished with Lipofectamine (Invitrogen), and cells were selected in hygromycin (50 μg/ml)-containing media. RAW 246.7 murine macrophages were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 200 units/ml penicillin, and 50 μg/ml streptomycin. Cells used for signaling experiments were serum-starved for 18 h before stimulation.

Isolation of Macrophages—Intraperitoneal macrophages were isolated according to methods reported previously (42). Briefly, CD36⁺/⁺ and CD36⁻/⁻ 3-month-old female mice (43) were injected with 2 ml of 4% Brewer thioglycollate intraperitoneally. After 4 days, the mice were sacrificed, and intraperitoneal macrophages were collected by lavage with cold PBS and further cultured in RPMI 1640 medium containing 10% fetal bovine serum for 24 h.

Arachidonic Acid and PGE₂ Release—Cells (~90% confluent in 6-well plates) were labeled with 0.5 μCi of [³H]arachidonic acid/well overnight. After washing to remove unincorporated label, the cells were incubated with or without racemic BEL or the cPLA₂α-specific inhibitor PYR and then stimulated for 5 min with 5 μM thapsigargin or 100 μM UTP. Lipids from the media and the cells were extracted and separated by TLC, and the zone containing fatty acids was scraped for scintillation counting (44). Prostaglandin E₂ release was measured using a PGE₂ Express EIA kit (Cayman Chemical Co.).

Measurement of Intracellular Ca²⁺—CHO cells or isolated intraperitoneal macrophages were grown in optical dishes or plates, and intracellular calcium concentration was measured according to a previously described protocol (45). Fura-2/AM or Fluo-4/AM (46) was used at a concentration of 2.5 or 1 μM, respectively.

siRNA Construction and Transfection—siRNAs directed against mouse CD36 were obtained from Invitrogen (Silencer® Select siRNA). The sequence specific for mouse CD36 was 5’-AACCCAGATGAGTGGCAA-3’. Transfection of siRNA (20 nm final concentration) in RAW 246.7 macrophages was performed using PepMute™ siRNA transfection reagent (SignaGen Laboratories, Ijamsville, MD) according to the manufacturer’s instructions. The siRNA against green fluorescent protein (47) was the negative control. In each case, the transfection medium was replaced with growing medium 24 h after transfection, and experiments were then performed 72 h after transfection.

Microscopy—Cells were grown on coverslips in standard media. For visualization of cPLA₂α and Fyn, cells were fixed in ice-cold methanol for 2 min and then in 3% formaldehyde for 15 min, stained with cPLA₂α or Fyn antibody for 1.5 h, washed five times with cold PBS, stained with a secondary antibody Alexa 594 (Invitrogen) for 1.5 h, washed five times, mounted, and visualized by confocal microscopy (48). For CD36 trafficking after thapsigargin treatment, live cells were stained at 4 °C with CD36 antibody for 1 h with CD36 antibody for 1 h, washed twice with cold PBS, and then stained at 4 °C with secondary antibody Alexa 594 (Invitrogen) for 40 min. The labeled cells were incubated at 37 °C for 10 min with or without 5 μM thapsigargin, fixed in 3% formaldehyde, and visualized by confocal microscopy.

Preparation of Membrane Fractions—Analysis of cPLA₂α translocation in the membrane fraction was performed as described previously (49). Briefly, cells were lysed in 0.25 M sucrose buffer with 1 mM deoxycholate and homogenized, and cell nuclei were removed by centrifugation (1,000 × g, 4 °C, 10 min). The supernatant was centrifuged for 1 h at 44,000 rpm.
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**Preparation of Triton-insoluble Membranes**—Separation of Triton X-100-insoluble membrane complexes was performed as described previously (50). A discontinuous gradient of 33, 20, and 5% sucrose was overlaid above cells in 40% sucrose. This was followed by centrifugation (5 h, 47,000 rpm, ~146,698 × g, 4 °C) in a TLS-55 rotor (Beckman Coulter, Brea, CA). Twelve fractions (200 μl) were collected from the top to the bottom of the tube, and aliquots were analyzed by Western blotting.

**Immunoprecipitation and Western Blot Analysis**—Whole cell extracts were prepared using a lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM pyrophosphate, 1 mM glycerol 2-phosphate) containing a protease inhibitor mix (Sigma-Aldrich). The cell lysates were clarified by centrifugation (12,000 × g, 10 min, 4 °C). All samples were separated on a 4–20% gradient SDS-polyacrylamide gel and electroblotted onto Immobilon-P polyvinylidene difluoride membranes. The membranes were blocked in phosphate-buffered saline, pH 7.5, 0.1% Tween 20 (PBST) with 50% Odyssey blocking buffer (LI-COR, Lincoln, NE), incubated with primary antibodies in PBST/Odyssey blocking buffer (1:1, overnight at 4 °C), and then incubated with infrared fluorophore-coupled secondary antibodies. The proteins were visualized using direct infrared fluorescence detection (Odyssey imaging system, LI-COR).

**Biotinylation of Cell Surface Proteins**—Cell surface proteins were labeled with biotin using EZ-Link® Sulfo-NHS-SS-biotin (Pierce, Rockford, IL) according to the manufacturer’s instructions. Cells were extracted using lysis buffer, and labeled proteins were pulled down using a Dynabeads® M-280 streptavidin kit (Invitrogen). The amounts of CD36 protein and the control surface marker Na+/K+ -ATPase were evaluated by Western blotting and quantified by densitometry.

**Real-time PCR Analysis**—Total RNA was isolated from tissues and cells using TRIzol (Invitrogen) as recommended by the manufacturer. RNA pellets were washed in 75% ethanol, dried at room temperature, and resuspended in UltraPure distilled water (Invitrogen), and the amount of RNA was quantified by spectrophotometry. Samples were amplified using the SuperScript III Platinum SYBR Green one-step quantitative RT-PCR kit (Invitrogen) on the SmartCycler system (Cepheid, Sunnyvale, CA). Results were analyzed by comparing the threshold crossing (Ct) of each sample after normalization to control genes (ΔCt). Changes in the threshold crossing (ΔCt) were used to calculate relative levels of each mRNA using the formula 2−ΔCt. The intron-spanning primer pairs used for amplification were as follows: cyclooxygenase-1 (Ptgs1), TGGGCTCACAAGGTACACGCTA and CACAGCCACATGCAGAACAT; cyclooxygenase-2 (Ptgs2), CTACAGAAGGAACCTCAGACT and TAGAATCCAGTCCGGGTACAGT. 36B4 was used as a real-time PCR control using the primers GCAGACAACGTGCTGATGATG and CACAGCCACATGCAGAACAT.

**Statistical Analysis**—Statistical analyses were performed using the GraphPad Prism 4 t test (two-tailed), and differences were considered significant at p ≤ 0.05.

**RESULTS**

**Arachidonic Acid Release Is Facilitated by CD36**—CHO cells have been used previously to dissect the pathways involved in AA release and eicosanoid formation (51–53). We generated CHO cells stably expressing human CD36 or containing an empty vector control. The cells were prelabeled with [3H]arachidonic acid overnight and then treated briefly (5 min) with thapsigargin (5 μM) to induce AA release. Thapsigargin depletes ER calcium stores by inhibiting the Ca2+ -ATPase pump (54), causing an increase in cytosolic calcium. Concomitant activation of plasma membrane calcium channels and influx of calcium result in activation of cellular phospholipases and subsequent release of AA from membrane phospholipids (45). As shown in Fig. 1A, thapsigargin treatment of CHO cells stably expressing CD36 resulted in a severalfold enhancement of AA release as compared with thapsigargin-treated cells transfected with an empty vector. To val-

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**Figure 1.** CD36 facilitates arachidonic acid liberation by phospholipases A2. A, enhanced AA release is CD36-dependent. CHO cells stably expressing wild-type human FLAG-CD36 (WT CD36) or empty vector (Vector) were labeled overnight with [3H]arachidonic acid. Mock-transfected or CD36-transfected CHO cells incorporated exogenous arachidonic acid to a similar degree (data not shown). The cells were stimulated with 5 μM thapsigargin (TG) for 5 min. Released arachidonic acid was determined in unstimulated (BG) cells, thapsigargin-stimulated cells, or cells pretreated either with 25 μM CD36 inhibitor S50, with the phospholipase A2, inhibitor BEL (10 μM), or with the cPLA2 inhibitor PYR (1 μM) for 15 min prior to thapsigargin stimulation, as indicated. CHO cells transfected with empty vector were used as controls. B, CD36 modulates PGE2 production. PGE2 production was measured in unstimulated cells after 10 min of thapsigargin treatment in CHO cells stably expressing wild-type human FLAG-CD36 or empty vector using a PGE2 enzyme immunoassay kit. The data are from three observations shown as means ± S.E. (error bars) and were validated in three independent experiments. *p < 0.05.
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idate the specificity of this effect with respect to CD36 expression, we used the CD36 inhibitor SSO, which covalently modifies and inhibits CD36 irreversibly (40). In cells treated with SSO (25 μM, 15 min) before the addition of thapsigargin, AA release was markedly inhibited, supporting the notion that the observed enhancement of AA release is CD36-dependent.

To establish that the AA liberated in the presence of CD36 originates from phospholipase A₂ activation by thapsigargin, the cells were treated with the phospholipase inhibitor BEL (10 μM) or PYR (1 μM), to inhibit the calcium-independent phospholipase A₂ (55) and cPLA₂α (56) enzymes, respectively, known to be expressed in CHO cells (57). As shown in Fig. 1A, both inhibitors partially decreased the amount of liberated [³H]arachidonic acid, suggesting the involvement of both phospholipases in AA release. Because the more marked effect of PYR (80%) was consistent with the interpretation that the AA release measured in these cells predominantly reflected activation of cPLA₂α, subsequent experiments were focused on this enzyme.

Liberated AA that is not released from cells or re-esterified serves as a precursor of eicosanoids. Measurement of the PGE₂⁺, a major product of AA oxidation, that is produced from CHO cells treated with thapsigargin showed severalfold more PGE₂ in the medium of CD36-transfected cells as compared with cells transfected with empty vector (Fig. 1B). These data suggested that CD36 expression strongly promotes AA release, which is coupled to PGE₂ production.

Thapsigargin Treatment Prevents CD36 Internalization—Previous work has demonstrated that CD36 cycles between intracellular compartments and the plasma membrane (3, 4). We examined whether thapsigargin altered the intracellular trafficking of CD36 by immunohistochemical localization studies. CHO cells were labeled with CD36 antibodies at 4 °C and then warmed to 37 °C to visualize CD36 internalization. As shown in Fig. 2A (left), thapsigargin treatment prevented internalization of CD36, which remains localized at the plasma membrane. Previously, the carboxyl terminus of CD36 has been demonstrated to be involved in signal transduction (58) and SFK activation (33, 59, 60). Moreover, two lysines in the carboxyl terminus (Lys⁴⁶⁹ and Lys⁴⁷²) were recently shown to be involved in signal transduction (58) and membrane. Previously, the carboxyl terminus of CD36 has been shown to be involved in signal transduction (58) and membrane localization of CD36, which remains localized at the plasma membrane (3, 4). Thus, we generated a line of CHO cells that stably express a mutated form of human CD36 where the two lysines were substituted with alanine (CD36 K/A). We then examined the effect of thapsigargin on the localization of this CD36 ubiquitination mutant. As shown in Fig. 2A (right), CD36 K/A localized to the plasma membrane in a manner similar to WT CD36 under control conditions. However, it was internalized in response to cell warming both in the absence and presence of thapsigargin. Thus, the effect of thapsigargin to block CD36 internalization was blunted with mutated K/A CD36.

To further confirm that thapsigargin increases the content of plasma membrane-localized CD36, biotinylation of surface proteins from cells treated with or without thapsigargin was conducted, followed by a pull-down assay using streptavidin beads and Western blotting. As shown in Fig. 2B, thapsigargin significantly increased the amount of cell surface-exposed CD36 in CD36 WT cells and failed to do so in CD36 K/A cells.

Because mutation of the lysine residues critical for the ubiquitination of CD36 inhibited the thapsigargin-induced retention of the protein at the plasma membrane, we examined whether CD36 K/A maintained the ability to mediate AA release. As shown in Fig. 2C, thapsigargin-induced AA release in CD36 K/A cells was significantly decreased in comparison with WT CD36. These data suggest that membrane localization of CD36 is important for its ability to facilitate AA release. Cells expressing the CD36 K/A ubiquitination mutant were used in subsequent experiments as a control for the specificity of the signaling events leading to AA liberation that were observed in WT CD36 cells.

CD36 Is Involved in the Regulation of Calcium Influx—Release of arachidonic acid from CHO cells was previously shown to be calcium-dependent (61). Therefore, we examined whether the thapsigargin-induced AA release facilitated by CD36 was sensitive to the presence of extracellular calcium. Inclusion of 2 mM EGTA in the medium to chelate extracellular calcium during thapsigargin stimulation completely abrogated arachidonic acid release from CHO cells expressing CD36 (Fig. 2D).

Thapsigargin induces ER calcium depletion, which triggers calcium influx via membrane store-operated calcium channels. We investigated the effect of the presence of CD36 on cellular calcium signaling in response to thapsigargin in the absence or presence of extracellular calcium. CHO cells expressing WT CD36 were loaded with Fura-2/AM, and changes in intracellular calcium concentration were monitored following treatment with thapsigargin in the presence or absence of the CD36 inhibitor SSO (Fig. 2E). Release of calcium from intracellular stores in response to thapsigargin in the absence of extracellular calcium (plus EGTA) was not influenced by pretreatment with SSO. However, inhibition of CD36 by SSO abrogated calcium influx after the addition of extracellular calcium. We also compared the intracellular calcium response to thapsigargin treatment in CHO cells expressing the CD36 K/A mutant in comparison with WT CD36. As shown in Fig. 2F, the response of cellular calcium to thapsigargin in the absence of extracellular calcium (plus EGTA) was comparable for the two cell types. However, upon the addition of extracellular calcium, the kinetic rates of calcium entry were different in cells expressing the CD36 K/A mutant (Fig. 2F) in comparison with WT CD36 controls. CD36 WT cells exhibited a sustained phase of calcium entry, whereas cytosolic calcium decreased with time in CD36 K/A cells. Taken together, the data in Fig. 2, D–F, support involvement of CD36 in the regulation of membrane calcium influx in response to calcium release from ER stores.

Activation of cPLA₂α—Cytosolic PLA₂α plays a major role in AA release and is activated by a sustained rise in intracellular calcium subsequent to calcium influx (18, 27, 62). Activation of cPLA₂α involves phosphorylation of the enzyme, which enhances its activity and its translocation to the ER, Golgi, and nuclear membranes, where it hydrolyzes arachidonate-containing phospholipids to release AA (28, 29). We first examined the effect of CD36 on cPLA₂α translocation by comparing the cPLA₂α content of membrane fractions prepared by differen-
FIGURE 2. CD36 membrane localization and calcium signaling in CHO cells. A, thapsigargin (TG) promotes CD36 membrane localization. CHO cells stably expressing FLAG-CD36 wild type (WT) or mutated CD36 with alanine substitutions at lysine 469 and lysine 472 (K/A) were labeled on ice with an anti-CD36 antibody and probed with an immunofluorescent secondary antibody. Subsequently, the cells were warmed to 37 °C for 10 min to induce internalization of CD36 (WARM) in the presence or absence of 5 μM thapsigargin (WARM+TG) and then fixed. Controls (CTRL) were fixed on ice without warming. B, surface biotinylation of CHO cells. CHO cells stably expressing FLAG-CD36 wild type and K/A were incubated in control medium (C) with or without 5 μM thapsigargin for 30 min. Cell surface proteins were then biotinylated, the cells were lysed, and biotinylated proteins were isolated using streptavidin beads. Proteins eluted from the streptavidin beads were used for Western blotting and probed with antibodies against CD36 and Na+/K+-ATPase. Densitometric analyses were performed, and results were calculated relative to control and Na+/K+-ATPase band intensities (n = 6 from three separate experiments). C, decreased AA release from cells expressing K/A CD36. WT and K/A cells were stimulated with 5 μM thapsigargin for 5 min, and released arachidonic acid was determined in unstimulated (BG) or stimulated (TG) cells or in cells pretreated with 25 μM SSO for 15 min before thapsigargin stimulation, as indicated. D, AA release is calcium-dependent. EGTA (2 mM) was used to chelate extracellular calcium during thapsigargin stimulation. The data (means ± S.E. (error bars), n = 3) were validated in three independent experiments. *, p < 0.05. E and F, calcium signaling is modulated by CD36. E, representative intracellular calcium signaling profiles of CD36 WT cells treated with thapsigargin in the presence or absence of 25 μM SSO. Calcium (1.8 mM final concentration) was then added to the medium (as indicated). F, CHO cells expressing wild-type CD36 or the CD36 mutant K/A were initially stimulated with thapsigargin (as indicated) in the absence of extracellular calcium prior to the addition of calcium to the medium. Cells expressing empty vector showed a response identical to CD36 K/A cells (not shown). The data are representative of four different experiments.
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FIGURE 3. Translocation of cPLA$_2$$\alpha$ to cellular membranes in response to thapsigargin (7G) is dependent on CD36. A, translocation of cPLA$_2$$\alpha$ to intracellular membranes. CHO cells expressing either wild-type CD36 or K/A CD36 in control medium (C) or stimulated with 5 $\mu$m thapsigargin for 30 min were lysed in sucrose/denyoxycholate buffer. Proteins in the membrane fractions were separated by centrifugation, subjected to SDS-PAGE, transferred to PVDF membranes, and probed with antibodies against cPLA$_2$$\alpha$, the ER marker calnexin, and $\beta$-actin. The graph shows densitometric analysis of the immunoblots. The data are representative of three separate experiments (means $\pm$ S.E. (error bars)). *, $p < 0.05$. B, confocal microscopy of cPLA$_2$$\alpha$ translocation. Cells grown on coverslips were treated as described above, fixed, and labeled with cPLA$_2$$\alpha$ (data are representative of three experiments).

FIGURE 4. Thapsigargin and UTP induce phosphorylation of ERK1/2 and cPLA$_2$$\alpha$. A, Western analysis profiles. CHO cells stably expressing FLAG-CD36 WT, CD36 K/A, or empty vector (Vector) were serum-starved overnight, kept in control medium (C), stimulated with 5 $\mu$m thapsigargin (TG) or 100 $\mu$m UTP in PBS for 10 min, and lysed. The clarified lysates were used for Western blotting and probed with antibodies against CD36, phospho-ERK1/2 (p-ERK1/2), ERK1/2, phospho-cPLA$_2$$\alpha$ (p-cPLA$_2$$\alpha$) Ser$^{505}$, and cPLA$_2$$\alpha$, as indicated. The data are representative of three separate experiments. B, calcium signaling mediated by UTP. CHO cells expressing WT or K/A CD36 were stimulated with UTP (as in Fig. 2). The data are representative of three different experiments.

The adenine nucleotide UTP is released into the extracellular milieu by cells responding to stresses such as mechanical stretch, hypoxia, or pathogen infection (63, 64). UTP mediates its effects on neighboring cells via membrane purinergic receptors and the inositol triphosphate pathway to release ER calcium, which is then followed by calcium influx through ion channels in the plasma membrane. Among the cellular events induced by UTP are activation of cPLA$_2$$\alpha$ and PGE$_2$ formation (63, 64). These effects contribute to the fine tuning of the inflammatory response via cell-to-cell communication (65). Thus, UTP and thapsigargin induce ER calcium depletion via distinct pathways, although they converge on activation of cPLA$_2$$\alpha$ via the MAPK ERK1/2 (64). UTP signaling is functional in CHO cells, which have endogenous purinergic receptors (66), so we examined the importance of the presence of CD36 for ERK1/2 and cPLA$_2$$\alpha$ phosphorylation after either thapsigargin or UTP treatment.

CHO cells expressing wild type or K/A CD36 were serum-starved for 18 h and stimulated with thapsigargin (5 $\mu$m) or UTP (100 $\mu$m) for 10 min, and phosphorylation of ERK1/2 and cPLA$_2$$\alpha$ was examined. As shown in Fig. 4A, increased phosphorylation of ERK1/2, which phosphorylates and activates phospholipase A$_2$ (38, 39), was observed after either thapsigargin or UTP treatment. In contrast, phosphorylation of ERK1/2 was absent or largely attenuated in cells containing the empty vector control or the CD36 K/A mutant, respectively. Consistent with this, robust phosphorylation of the ERK1/2 target residue Ser$^{505}$ of cPLA$_2$$\alpha$ was observed in cells expressing WT CD36. These data support the interpretation that thapsigargin and UTP activate a CD36-dependent MAPK signaling pathway, which phosphorylates and activates cPLA$_2$$\alpha$.

Next, we sought to determine whether UTP exerted similar effects on calcium signaling as thapsigargin in cells expressing either WT or K/A CD36. As shown in Fig. 4B, UTP rapidly increased intracellular free calcium released from the ER into the cytosol, followed by its rapid sequestration in cells expressing either WT or K/A CD36. However, as with thapsigargin treatment, sustained calcium entry was attenuated in CD36 K/A cells, where cytosolic calcium decayed more rapidly than in WT cells. These data, together with the results in Fig. 2, suggest that CD36 participates in the regulation of calcium influx in...
CD36-expressing cells were grown on coverslips and labeled with Fyn antibody or Fyn kinase. Calcium influx was measured in the presence or absence of the SFK inhibitor Src-I1. Calcium influx was increased in WT-expressing cells stimulated with thapsigargin in the presence of K/A CD36 WT and K/A CD36 expressing WT or K/A CD36 with Fyn was similar in cells expressing WT or K/A CD36 (not shown). In contrast, Fyn distribution was altered. Specifically, the kinase was absent from the high density soluble fractions of K/A CD36 lysates, whereas it was present in the corresponding fractions from WT CD36 cells (Fig. 5C). This was quantified by densitometric analysis (Fig. 5D). The altered distribution of Fyn was further confirmed by fluorescent confocal microscopy of cells labeled with anti-Fyn antibody. An increased intensity of Fyn labeling at the cell periphery in CD36 K/A cells was manifest as compared with WT-expressing cells (Fig. 5, C–E). These data indicate that in cells expressing K/A CD36, cellular distribution of Fyn is altered despite no significant differences in its phosphorylation state.

**Primary and RAW 246.7 Macrophages**—Liberation of AA driven by calcium influx is important for eicosanoid formation in monocytes and macrophages, which express high levels of CD36. To determine whether the observations in CHO cells were applicable to leukocytes, mouse intraperitoneal macrophages were isolated from CD36+/+ and CD36−/− mice, and release of AA was measured after 10 min of thapsigargin or UTP stimulation. Liberation of AA after 10 min was significantly lower in CD36−/− macrophages as compared with CD36+/+ cells after either treatment (Fig. 6A). Both thapsigargin and UTP enhanced phosphorylation of ERK1/2 and cPLA2 in CD36+/+ macrophages as in CHO cells, and these effects were markedly attenuated in CD36−/− macrophages (Fig. 6B).

The increases in cytosolic calcium in response to thapsigargin in the presence of EGTA in the medium followed by the addition of extracellular calcium were examined in macrophages from both genotypes. As shown in Fig. 6C, CD36−/− macrophages exhibited a significantly reduced response of intracellular calcium to thapsigargin both in the presence and absence of EGTA in comparison with WT macrophages.

To further verify the role of CD36 in cPLA2 activation in macrophages, the direct effect of CD36 knockdown in RAW 246.7 murine macrophages on cPLA2 activation was examined. As shown in Fig. 6D, approximately ~50% down-regulation of CD36 expression was obtained with siRNA against CD36. Down-regulation of CD36 resulted in blunted phosphorylation of cPLA2 in response to either thapsigargin or UTP. Fig. 6E shows that the addition of UTP or the calcium ionophore A23187 to RAW 246.7 macrophages increased plasma membrane CD36 content as determined by biontinyla- tion of surface proteins using Sulfo-NHS-SS-biotin. Importantly, the data with primary and RAW 246.7 macrophages are consistent with earlier data in CHO cells.
immunologic cells, we examined the ability of primary macrophages isolated from WT and CD36-deficient mice to produce prostaglandin E2. The isolated macrophages were treated with thapsigargin, and production of PGE2 was measured and compared with that of WT cells assayed under the same conditions. In contrast to the results with CHO cells expressing CD36, macrophages null for CD36 produced more PGE2 in response to thapsigargin as compared with WT macrophages (Fig. 7A).

Recent evidence suggests that long term changes in the level of free AA induce compensatory increases in PGE2 formation (26), which may be related to regulation of enzymes involved in AA oxidation. We examined whether expression of cyclooxygenase 1 (Cox1 or Ptgs1) and 2 (Cox2 or Ptgs2) and of lipoxygenase-5, enzymes involved in PGE2 generation, was altered in CD36+/− mice compared with CD36−/− macrophages. Messenger RNA levels as determined by RT-PCR were increased for Cox-1 and Cox-2 by 4- and 12-fold (Fig. 7A), respectively, whereas lipoxygenase-5 levels were unchanged (data not shown). Western analyses revealed that protein levels for the cyclooxygenases were also increased in parallel with their mRNA levels. Cox-1 and Cox-2 protein levels were increased by 30 and 450%, respectively, in CD36+/− macrophages relative to their CD36−/− counterparts (Fig. 7C).

Collectively, these data suggest that the reduced flux of AA release in CD36−/− cells may induce long term compensatory up-regulation of cyclooxygenase expression and PGE2 production in macrophages.

**DISCUSSION**

In this study, we demonstrate that CD36 participates in the regulation of cellular calcium influx in response to depletion of ER calcium stores triggered either via inhibition of SERCA (sarco/endoplasmic reticulum Ca2+/ATPase) or by purinergic receptor activation. Moreover, our results show that an important consequence of this role of CD36 is activation of the cellular events that result in AA release including cyclooxygenase and eicosanoid formation.

**CD36 Modulates Calcium Entry in Response to ER Calcium Depletion**—Store-operated or receptor-operated calcium channels in the plasma membrane (PM) are activated by depletion of intracellular calcium stores to promote calcium influx, result-
The role of CD36 in calcium dynamics remains at the earliest stages of understanding. In isolated hypothalamic ventromedial nucleus neurons, the addition of oleic acid resulted in alterations of intracellular calcium oscillations, and these effects were reduced by the CD36 inhibitor SSO (67). More evidence for a link between CD36 and cellular calcium was obtained using taste bud cells sorted based on CD36 expression (68). It was shown that exposure of CD36-positive cells to extracellular linoleate increased intracellular calcium through 1,4,5-trisphosphate-mediated release of ER calcium. In the presence of factors (3, 4). Our data suggest that signals that induce release of ER calcium promote CD36 membrane localization. The ubiquitination mutant of CD36 (K/A) in CHO cells to link the protein to calcium dynamics. In addition, regardless of whether ER calcium depletion was initiated at the ER by thapsigargin or at the plasma membrane by UTP, CD36 was found to modulate the activation of PM calcium channels, supporting its involvement in the maintenance of intracellular calcium stores. Intracellular calcium signaling regulates a wide range of cellular functions, including contraction, secretion, and metabolism. Whether the activation of calcium influx channels is important for CD36 function in phagocytosis, apoptosis (2, 8), and thrombus formation (6, 69) is unknown and requires further study.

**CD36 Enhances AA Release**—Among the consequences of the rise in intracellular calcium in cells expressing cytosolic PLA2, the stimulation of AA release from the sn-2 position of phospholipids and its use for synthesis of eicosanoids (70), which are potent mediators of a wide range of proinflammatory effects (19–21). Cellular arachidonic acid (20:4n-6) derived from dietary sources or synthesized from linoleic acid can constitute up to 25% of the fatty acyl moieties present in phospholipids. Arachidonic acid release is tightly regulated by PLA2 enzymes, which are activated through a variety of surface receptors for hormones and cytokines (71). Cytosolic PLA2 plays a major role in AA release through its calcium-dependent translocation to cellular membranes to hydrolyze AA esterified to membrane phospholipids. The activity of cPLA2 is also enhanced by MAPK-dependent phosphorylation (18). Our results show that the CD36-dependent AA release was decreased by 80% by the cPLA2 inhibitor PYR. In addition, chelation of extracellular calcium by EGTA completely abolished AA release, indicating that it was calcium-dependent and thereby consistent with cPLA2 activation. Furthermore, cPLA2 translocation and phosphorylation were blunted in cells expressing mutated K/A CD36, which were deficient in their ability to release AA.

The trafficking of CD36 between the plasma membrane and multiple intracellular compartments is regulated by a number of factors (3, 4). Our data suggest that signals that induce release of ER calcium promote CD36 membrane localization. The ubiquitination mutant of CD36 (K/A) used in the current study, which is resistant to thapsigargin-induced membrane recruitment, was inefficient in enhancing calcium influx, cPLA2 phosphorylation, or AA release. Plasma membrane localization of CD36 is probably important for the CD36-associated Fyn kinase to access and regulate the opening of PM calcium channels (30). Although we could not detect differences in Fyn phosphorylation in cells expressing WT versus mutated CD36, the altered localization of CD36-Fyn in CD36 K/A-expressing cells provides a potential mechanism for the inability of this mutant to induce calcium entry and AA release.
The findings in this study suggest that the role of CD36 in inflammation may in some cases be related to its regulation of AA release. In agreement with the results utilizing CHO cells, we found that thapsigargin- or UTP-triggered calcium signaling, phosphorylation of ERK1/2 and cPLA2α, and subsequent AA release were impaired in mouse intraperitoneal macrophages isolated from CD36−/− as compared with WT mice. In addition, siRNA knockdown of CD36 in mouse RAW 264.7 macrophages led to a decrease of cPLA2α phosphorylation. The liberation of AA from arachidonate-containing phospholipids in monocytes and macrophages is physiologically important during inflammation and determines the rate of synthesis of eicosanoids, which have potent proinflammatory actions and are implicated in multiple aspects of cell physiology (19–23). The data in Fig. 7 showing enhanced PGE2 production by CD36−/− macrophages despite reduced AA release suggest that cyclooxygenase expression and resultant PGE2 production may be regulated by the availability of free AA. Despite the decrease in thapsigargin-induced AA release from CD36−/− macrophages, production of PGE2 was enhanced, apparently as a result of significantly increased expression of Cox-2 and, to a lesser extent, Cox-1. Thus, chronic CD36 ablation resulted in a compensatory response to the defect in the AA liberation through the increased production of Cox-2. Consistent with these findings, it was recently shown in vascular cells that although acute down-regulation of ACSL4 (acyl-CoA synthetase 4), which selectively acylates AA to AA-CoA for esterification, resulted in increased PGE2 release, the opposite response occurred (i.e., a reduction in PGE2 release) with stable, long-term reduction of ACSL4 (26).

In summary (Fig. 8), CD36 modulates the pathways that maintain calcium homeostasis in response to depletion of ER calcium. One aspect of this role involves cPLA2α activation and liberation of arachidonic acid for eicosanoid formation. Eicosanoids are mediators of pleiotropic inflammatory effects and have been linked to multiple pathologies (19–23), including hypertension (72) and coronary heart disease (73). Polymorphisms in the cyclooxygenase 2 gene have been associated with susceptibility to stroke and myocardial infarction (74, 75), with type 2 diabetes (76), with coronary artery disease (77), and with cancer (78). Further studies of how this novel function of CD36 integrates the complex mechanisms that regulate cellular calcium and those involved in eicosanoid formation will be important in the understanding of the etiology of inflammatory processes and their contribution to disease.

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