Published in final edited form as:

Biomarkers. 2018 December ; 23(8): 756–765. doi:10.1080/1354750X.2018.1490968.

Extracellular esterase activity as an indicator of endoplasmic reticulum calcium depletion

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Abstract

Context—Endoplasmic reticulum (ER) calcium depletion is associated with diverse diseases, including cardiac, hepatic and neurologic diseases.

Objective—The aim of the present study was to identify and characterize an endogenous protein that could be used to monitor ER calcium depletion comparably to a previously described exogenous reporter protein.

Materials and methods—The use of a selective esterase-fluorescein diester pair allowed for carboxylesterase activity in extracellular fluid to be measured using a fluorescent readout. Cell culture media from three different cell lines, rat plasma, and human serum all possess quantifiable amounts of esterase activity.

Results—Fluorescence produced by the interaction of carboxylesterases with a fluorescein diester substrate tracks with pharmacological and physiological inducers of ER calcium depletion. The fluorescence measured for *in vitro* and *in vivo* samples were consistent with ER calcium depletion being the trigger for increased esterase activity.

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Disclosures

The authors have no disclosures.

Discussion—Decreased luminal ER calcium causes ER resident esterases to be released from the cell, and, when assessed concurrently with other disease biomarkers, these esterases may provide insight into the role of ER calcium homeostasis in human diseases.

Conclusion—Our results indicate that carboxylesterases are putative markers of ER calcium dysfunction.

Keywords

Carboxylesterase; liver esterase; endoplasmic reticulum; calcium; SERCaMP; ER homeostasis

Introduction

Calcium levels in the endoplasmic reticulum (ER) lumen are over one thousand times higher than in the cytosol. Maintaining a steep calcium gradient is critical for cell signaling, drug detoxification, and protein synthesis. ER calcium depletion is implicated in numerous pathologies including cardiovascular disease, diabetes, and cancer (Mekahli et al., 2011). Although changes in ER calcium may be a contributing pathological mechanism to disease or occur as a symptom of disease, our limited ability to monitor ER calcium prevents further advancements in our understanding of the role of ER calcium in this regard.

In cell culture models, calcium sensitive dyes, fluorescent calcium indicators, and genetically encoded calcium indicators have been used to assess ER calcium levels (Palmer et al., 2004, Rehberg et al., 2008). However, these techniques are restricted to acute measurements and are not readily applicable to longitudinally monitoring ER calcium. We previously developed a Gaussia luciferase (GLuc) based calcium reporter as an alternative method to monitor ER calcium depletion (Henderson et al., 2014, Henderson et al., 2015). This reporter, GLuc-ASARTDL, utilizes the seven terminal amino acids of mesencephalic astrocyte derived neurotrophic factor (MANF) appended to GLuc to localize the reporter to the ER lumen and cause its release in response to decreased luminal ER calcium. While GLuc-ASARTDL offers a method of assessing ER calcium depletion over time, limitations arise when adapting GLuc-ASARTDL assays for *in vivo* use because of the need to employ transgenic techniques to provide the reporter.

The seven amino acid C-terminal tail of MANF, ASARTDL, is structurally and functionally similar to the canonical ER retention signal KDEL. Previous studies found that other permutations to the KDEL signal can act as ER retention motifs as well (Raykhel et al., 2007, Mei et al., 2017). Given that ER calcium depletion triggers the release of GLuc-ASARTDL from the ER, it is plausible that an endogenous ER resident protein with a similar C-terminal tail could be secreted in response to ER calcium depletion and measured in extracellular fluid. Our goal was to identify an endogenous ER resident protein with a KDEL-like carboxy terminal tail that is secreted in response to ER calcium depletion and can be measured *in vitro* and *in vivo* without introducing foreign agents into cells or animals. The present study proposes that carboxylesterases can be used as a putative endogenous biomarker of ER calcium depletion. Based on a selective esterase-fluorescein diester pair identified by Tian et al. (2012), we developed a fluorescent based assay to

measure esterase activity *in vitro* and *in vivo* and monitor changes in ER calcium homeostasis.

Materials and Methods

Materials and reagents

Chemicals used (all diluted in DMSO) were: thapsigargin (Sigma), dantrolene (Sigma), cyclopiazonic acid (Sigma) loperamide hydrochloride (Cayman Chemical), 4-Nitrophenyl phenylphosphonate (Cayman Chemical), troglitazone (Cayman Chemical), benzil (EMD Millipore), tunicamycin (Sigma). DMSO controls at concentrations equivalent to treatments were used in all experiments. The preceding reagents are commercially available at the indicated companies. The plasmids and cell lines generated as part of this study are available by request from the authors.

Sequence alignment

EST1_PIG, EST1_HUMAN, and EST2_HUMAN were aligned using Uniprot sequence alignment with the Clustal Omega program. Annotations were added to show amino acid similarity.

Plasmid DNA

The coding region for porcine liver esterase with its signal peptide removed (comprising amino acids 19–566) was amplified from pCAG::PLE-IRES-mCherry (a gift from Dr. Scott Sternson) and inserted downstream of the MANF signal peptide (1–23) and the StrepII-EK-FLAG epitopes in the pLenti6.3 lentiviral plasmid backbone (Invitrogen) with ligation-independent cloning. An amino acid polymorphism (A439T) was discovered upon sequencing, and PCR-based mutagenesis was used to modify this codon back to A439V (with respect to the “wildtype” primary sequence for PLE Genbank CAA44929.1 and the digital sequence provided for AA0707) thereby creating pLenti6.3 hMANFsigpep-StrepII-FLAG PLE (Addgene 104482).

Cell culture and transfections

Human SH-SY5Y neuroblastoma cells were maintained in growth medium composed of Dulbecco's Modified Eagle Medium (DMEM 1X + GlutaMAX, 4.5 g/L D-glucose, 110 mg/L sodium pyruvate; Invitrogen) containing 10% bovine growth serum (BGS; GE Life Sciences SH30541.03), 10 units/mL penicillin, and 10 µg/mL streptomycin. Cells were grown at 37°C with 5.5% CO₂ in a humidified incubator. For PLE overexpression, cells were reverse transfected by adding cells to a 12-well plate containing transfection complexes. Transfection complexes contained 2 µg PLE plasmid DNA and 0.6 µL of Xfect (Clontech) in a final volume of 100 µL. Wells had a final volume of 1 mL and 5.9×10^5 cells in cell culture medium without antibiotics. After 28–29 h, a full medium exchange into growth medium containing 1.5% BGS was performed. This was followed 16 h later by a full medium exchange into esterase assay medium (150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 20 mM HEPES, 1 mM CaCl₂, and 1.9 g/L glucose). After 8 h medium was collected and snap frozen.

All other cell culture experiments were performed in 96-well plates with 5×10^4 cells in 100 μ L. After 28–29 h, a full medium exchange into low serum (1.5% BGS) growth medium was performed. Drug treatments (e.g. thapsigargin, CPA) began 16–17 h after the low serum media exchange and lasted for 8 h. At the time of drug treatment, a full media exchange was performed with esterase assay medium containing drug at 1x final concentration. For pre-treatments, dantrolene was added 30 min prior to SERCA inhibition by CPA. At the end of each experiment, medium was removed from the wells, spun down for 5 min (1000 xg, 4°C), and snap frozen. For CPA experiments, washouts were performed via six 50% media exchanges and occurred 2 h after the initial drug application. Media samples were collected 8 h after the final washout.

Human Hep3B and HepG2 hepatocyte cells were maintained similarly to SH-SY5Y (described above) but used DMEM containing 10% fetal bovine serum (FBS; GE Life Sciences SH30071.03), 10 units/mL penicillin, and 10 μ g/mL streptomycin. Low serum media exchanges in these cell lines were done with growth medium containing 1.5% FBS.

Viability assays

Cell viability assays were performed with the Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS assay) and Promega CellTiter-Glo Luminescent Cell Viability Assay (ATP assay) as per the manufacturer's protocol.

Cell viability was also measured using the Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS assay) by adding 20 μ L of CellTiter 96 Aqueous One Solution Reagent to each well of a 96-well plate containing 100 μ L of culture medium. The plate was incubated for 1 h at 37°C with 5.5% CO₂ then absorbance at 490 nm was measured with a Biotek Synergy II plate reader.

For the Promega CellTiter-Glo Luminescent Cell Viability Assay (ATP assay), the CellTiter Glo Reagent volume equal to the volume of cell culture medium was added to each well. Plates were mixed on an orbital shaker for 2 min at approximately 50 rpm then incubated for 10 min at room temperature. 100 μ L of the solution was transferred from the tissue culture plate to an opaque walled plate, and luminescence was measured with a BioTek Synergy II plate reader.

Oxygen glucose deprivation (OGD)

SH-SY5Y cells were maintained in culture medium (DMEM 1X + GlutaMAX, 4.5 g/L D-glucose, 110 mg/L sodium pyruvate, containing 10% FBS, 10 units/mL penicillin, and 10 μ g/mL streptomycin) prior to plating in 96-well plates. 28 h after plating, cells were exchanged into OGD medium. The OGD medium (culture medium without glucose) was deoxygenated by bubbling a gas mixture containing 5% CO₂, 10% hydrogen, and 85% nitrogen for 15 min (Goldberg and Choi, 1993). Every plate was exchanged into culture medium as described above (normoxia control) or OGD medium (DMEM with L-Glutamine containing 10% FBS, 10 units/mL penicillin, and 10 μ g/mL streptomycin). For dantrolene treatments, dantrolene (30 μ M) or vehicle was added directly into deoxygenated or normoxia media immediately prior to the media being added to the culture plate. OGD plates were placed into hypoxia chambers (Billups-Rothenberg) that were deoxygenated by flushing the

chambers for 10 min with 5% CO₂, 10% hydrogen, and 85% nitrogen. The hypoxia chambers were placed inside of a 37 °C incubator. After 16 h of oxygen and glucose deprivation, cells were removed from the hypoxia chambers and medium was replaced with glucose-containing esterase assay medium. All plates were returned to the incubator until it was time for sample collection.

Fluorescein diester assays

For fluorescein diester assays, 20–75 µL of cell culture medium or 20 µL of plasma was transferred to a black walled clear bottomed plate (Perkin Elmer). An equal volume of 100 µM esterase substrate, fluorescein di-(1-methylcyclopropanecarboxymethyl ether), was added to each well. The substrate was diluted in esterase assay medium (pH 5). Fluorescein di-(1-methylcyclopropanecarboxymethyl ether) was initially prepared at 100 mM in DMSO and stored at –80°C as single use aliquots. Diluted substrate was kept in dark conditions prior to use. Fluorescence was measured every minute for 60 min at 45°C using a BioTek Synergy H2 plate reader (Excitation 485 nm/Emission 528 nm). The fluorescent values presented are those read at the 1 h timepoint after substrate addition. For inhibitor assays, serum free cell culture medium or rat plasma were incubated with various concentrations of esterase inhibitors (listed above) for 30 min prior to running a fluorescein diester assay as described above.

Gaussia luciferase secretion assay

Assays for *Gaussia* luciferase activity were performed as previously described (Henderson et al., 2014, Henderson et al., 2015). For luciferase secretion assays, 5 µL of cell culture medium from each well was transferred to a new opaque walled plate. The GLuc substrate was 10 µM coelenterazine (Regis Technologies) in PBS. Coelenterazine stock solutions were prepared at 20 mM in acidified methanol (10 µL of 10 N HCl per 1 mL of methanol) and stored at –80°C as single use aliquots. Luciferase levels were determined using a plate reader with an injector setup (BioTek Synergy II) that allowed samples to be read directly after injecting 100 µL of substrate into the well containing cell culture medium. For all secretion assays, vehicle controls were used in all experiments at concentration equivalent to the treatments. For luciferase assays involving rat plasma, 10 µL of plasma was transferred to an opaque walled plate and 100 µL of PBS containing 100 µM coelenterazine and 500 mM ascorbic acid was injected. Cell culture samples were read at 25°C with a sensitivity of 100 and a 0.5 second integration time with a 5 second delay following coelenterazine substrate injection using a BioTek Synergy II plate reader. Rat plasma samples were read at 25°C with a sensitivity of 150 and a 5 second integration time with a 5 second delay following coelenterazine substrate injection using a BioTek Synergy II plate reader.

Real time reverse transcription quantitative PCR (RT-qPCR)

mRNA expression was measured by real time RT-qPCR. Total RNA was isolated from cells using the NucleoSpin® RNA kit (Takara) according to the manufacturer's protocol. Total RNA from cell samples was isolated using the NucleoSpin RNA kit (Macherey-Nagel). RNA concentration was quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA samples were stored at –80°C until cDNA synthesis.

Using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories), 1 µg of RNA sample was transcribed into cDNA in a 20 µL reaction mix according to the manufacturer's protocol and diluted 1:20 with DNase-free water. cDNA, along with appropriate primer/probe sets (final concentration 450 nM and 50 nM, respectively), and 2X Universal TaqMan Master Mix (Thermo Fisher Scientific) were amplified in a 20 µL reaction mix using BioRad CFX96 (5 min at 95°C, 50 cycles of 20 secs at 94°C, 1min at 60°C). Primer and probe sequences are listed in Table 1. Ct values were normalized to the housekeeping gene Ube2i (Kobayashi et al., 2004). Fold change was calculated using 2^{-Ct} and data are expressed as fold change with upper and lower limits ($2^{-Ct \pm SD}$).

Human blood samples

Blood samples were obtained from individuals who participated in a human laboratory study conducted at the National Institutes of Health Clinical Center in Bethesda, MD. This study was registered at [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01751386) (NCT01751386); the parent protocol and the use of blood samples for the present study were approved by the NIH Addictions Institutional Review Board. Briefly, enrolled participants fit the DSM-IV-TR criteria for a diagnosis of current alcohol dependence and high trait anxiety and were not seeking treatment for either condition. Further information as well as a detailed description of inclusion and exclusion criteria was previously reported (Farokhnia et al., 2017). The blood samples used in this study were from the participant's baseline visit (Day 1) after a written consent form was provided, but before the study medication or alcohol was administered. Breath alcohol concentrations at the time of this blood draw was zero. Age, body mass index (BMI), and markers of liver inflammation [i.e. aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] measures were available for all patients. C-reactive protein (CRP) concentrations, measured as a marker of general inflammation, were not available for all participants. Grubb's test for outliers was performed on all data and resulted in the exclusion of one participant from the AST/ALT analysis and one participant from the CRP analysis. Outliers were not included in our analyses.

In vivo injections

All animal procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the National Institute on Drug Abuse Intramural Research Program, Baltimore, MD. For intrahepatic injections of adeno-associated viral (AAV) vectors, male Sprague-Dawley rats were injected with AAV-GLuc-ASARTDL under anesthesia as described previously (Henderson et al., 2015, Wires et al., 2017). Briefly, a 30-gauge needle was used to inject 100 μ L of virus (7.6×10^9 vg/mL) into the medial lobe of the liver at 3 injections sites. Animals received an intraperitoneal (i.p.) injection of 1 mg/kg thapsigargin 14 days after viral injection and a vehicle i.p. injection 28 days after viral injection. Blood was sampled via tail snip at indicated times as described previously. Male Sprague-Dawley rats used for C-reactive protein (CRP) experiments were not injected with AAV and received a single i.p. injection of either 1 mg/kg thapsigargin or vehicle.

During blood collection, animals were anesthetized with isoflurane, tails were snipped, and blood was collected into tubes containing heparin (50 μ L of 1000U/mL heparin). The ratio of blood to heparin was normalized to 2:1 then samples were centrifuged at 4 C for 5 min at 2000xg to separate plasma. Plasma was stored at -80 C until time of use.

C-reactive protein ELISA

An ELISA for rat C-reactive protein was performed according to the manufacturer's protocol (Thermo Fisher Scientific). Rat plasma samples were diluted 1:4000 prior to assaying. Reaction with the provided tetramethylbenzidine substrate solution was allowed to proceed for 10 min before quenching with the kit's acidic stop solution. Absorbance at 450 nm was read using a BioTek Synergy II plate reader.

Albumin quantification

Rats were deeply anesthetized with isoflurane and blood was collected via cardiac stick 48 h post-Tg or vehicle injection into collection tubes provided by IDEXX laboratories (Westbrook, ME). Following collection, blood samples were centrifuged at 2000xg for 5 min at 4°C, and serum was transferred to a separate tube and stored at -80°C until time of shipment. Measurements for albumin were performed by IDEXX laboratories.

Results

Esterase-fluorescein diester assay

We sought to identify a secreted endogenous protein with an ER localization sequence and an enzymatic activity that could be exploited as a robust and sensitive reporter of ER calcium dysregulation like our previously described GLuc-SERCaMP reporter (Henderson et al., 2014). In examining the proteome for proteins with KDEL-like carboxy terminal tails, we found several esterases with the properties outlined above. We focused on carboxylesterases because of their known ability to hydrolyze a variety of esters, amides, and carbamates. A specific carboxylesterase-substrate interaction was previously described for porcine liver esterase (PLE) with a fluorescein-di-(1-methylcyclopropanecarboxymethyl ether) (fluorescein-CM2) substrate (Tian et al., 2012). Esterase activity on fluorescein-CM2 cleaves α -cyclopropyl ester linkage, followed by rapid, spontaneous release of formaldehyde and the phenolic fluorophore (Lavis et al., 2011, Tian et al., 2012). PLE has a KDEL-like C-terminus (KIKHAEL), which is similar in structure to the human esterases CES1 (C-terminal QTEHIEL) and CES2 (C-terminal EERHTEL; Figure 1). A boxshade plot comparing PLE to human CES1, and CES2 reveals that PLE is most similar in sequence to CES1 (Supplementary Figure 1). Based on previous work (Tian et al., 2012), we developed a plate reader assay to measure esterase activity and demonstrated that medium from SH-SY5Y cells transfected with PLE exhibited increased fluorescence compared to control transfected cells (Figure 2(a)). To optimize assay conditions, media from SH-SY5Y cells transfected with PLE was collected and tested at various pH, substrate concentrations, and temperatures (Figure 2(b-d)). Additionally, esterase activity was observed to be linear over four orders of magnitude (Figure 2(e)). Based on these studies, we selected assay conditions of 100 μ M fluorescein-CM2 substrate (pH 5.0, 45°C) and a sample volume ranging from 20 μ L to 75 μ L. Small molecule inhibitors of esterases were used to characterize the esterase signal attributable to PLE. The fluorescent signal was abolished in collected medium treated with the general carboxylesterase inhibitors 4-Nitrophenyl phenylphosphonate (BNPP) or benzil (Figure 2(f)) (Hatfield and Potter, 2011). Treatment with troglitazone (CES1 inhibitor) (Fukami et al., 2010) and loperamide (CES2 inhibitor) (Hatfield and Potter, 2011)

also diminished the fluorescent signal (Figure 2(f)). None of the inhibitors used exhibited fluorescence above background when measured alone (Figure 2(g)).

ER calcium depletion increases esterase activity

Thapsigargin (Tg) pharmacologically depletes ER calcium by inhibiting the sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) (Davidson and Varhol, 1995). Our SH-SY5Y neuroblastoma cell line that stably expresses GLuc-ASARTDL has been previously characterized as a reporter of ER calcium depletion (Henderson et al., 2014). Here, we show that an increase in extracellular luminescence from GLuc-ASARTDL following Tg treatment was accompanied by an increase in fluorescence from esterase activity (Figure 3(a)). Tg did not cause any significant change in cell viability (Supplementary Figure 2(a–b)). Given that CES1 is a liver carboxylesterase and CES1 inhibitors decreased the esterase signal in previously tested samples, endogenous esterase activity was further tested in two human hepatoma cell lines, Hep3B and HepG2. Tg significantly increased media esterase activity in both cell lines (Figure 3(b)). BNPP and benzil (pan-carboxylesterase inhibitors), troglitazone (CES1 inhibitor), and loperamide (CES2 inhibitor) dose dependently reduced esterase activity indicating a carboxylesterase is responsible for the fluorescent signal being produced and that CES1 and CES2 can hydrolyze the ester bonds of fluorescein-CM2 (Figure 3(c)).

Tg induces ER stress while causing ER calcium depletion while tunicamycin (an N-linked glycosylation inhibitor) induces ER stress without causing ER calcium depletion. Similar to the published GLuc-ASARTDL reporter (Henderson et al., 2014), only Tg caused an increase in fluorescent signal in Hep3B cells (Figure 4(a)). Induction of ER stress was confirmed by monitoring the upregulation of genes involved in the unfolded protein response (UPR). BiP, Erdj4, and ASNS are endogenous UPR-responsive genes activated by the ATF6, IRE1/XBP1, and PERK UPR pathways, respectively (Shoulders et al., 2013, Gjymishka et al., 2009). The mRNA levels of BiP, Erdj4, and ASNS were increased following treatment with both Tg and tunicamycin, however Tg caused a greater increase in UPR-responsive gene expression (Supplementary Figure 3(a)). When using the SH-SY5Y cell line in which Tg and tunicamycin induce similar increases in established ER stress markers, tunicamycin did not increase fluorescent esterase signal (Supplementary Figure 3(b–c)). In neither case did the drug treatments affect cell viability (Supplementary Figure 2(c–f)). Further, in Hep3B cells, the effect of Tg was dose-dependent (Figure 4(b)) and a similar effect was also observed with cyclopiazonic acid (CPA) (Goeger et al., 1988), a reversible SERCA inhibitor, (Figure 4(c)). Treatment with dantrolene, a ryanodine receptor inhibitor that blocks calcium efflux from the ER, attenuated the CPA evoked esterase release from Hep3B cells with minimal effects on cell viability (Figure 4(d), Supplementary Figure 2 (g–h)). A CPA and dantrolene washout paradigm was used to circumvent the interference of dantrolene fluorescence (Dehpour et al., 1982).

Oxygen-glucose deprivation (OGD) is used as an *in vitro* model of ischemic injury where changes in calcium homeostasis are known to occur (Badiola et al., 2011, Wang et al., 2002). SH-SY5Y cells were subjected to OGD for 16 h followed by medium replacement. Esterase activity increased in the medium following OGD injury (Figure 4(e)). Treatment with

dantrolene during OGD reduced the level of esterase-dependent fluorescence which further supports our model in which extracellular esterase tracks with ER calcium dysregulation caused by pathophysiological states.

In vivo esterase activity

To characterize esterase secretion *in vivo*, we concurrently monitored GLuc-ASARTDL and esterase activity in plasma. In order to monitor GLuc-ASARTDL activity *in vivo*, rats were intrahepatically injected with AAV-GLuc-ASARTDL as previously described (Henderson et al., 2015) and given Tg 14 days after viral infection. GLuc and esterase activity in plasma was significantly increased following Tg as indicated by increased luminescence and fluorescence, respectively (Figure 5(a)). No change in luminescence or fluorescence was observed following a vehicle injection. The Tg-induced increase in esterase activity was temporally consistent with increases in GLuc-ASARTDL with the greatest change seen in the 48 h following Tg. To examine the stability of esterase activity, plasma from animals was incubated for an additional 48 h at 37°C and showed a slight increase in fluorescence (data not shown) indicating that the return to baseline is unlikely due to esterase instability. Incubation of the rat plasma with carboxylesterase inhibitors produced the same pattern of inhibition seen previously in cell culture medium, supporting the idea that carboxylesterases are the enzymes responsible for fluorescein-CM2 substrate conversion with BNPP, benzil, troglitazone, and loperamide all decreasing esterase activity (Figure 5(b)).

Human blood samples from a clinical laboratory study (Farokhnia *et al.*, 2017) were examined for esterase activity. While no significant correlation was found between esterase activity and either age or body mass index (BMI), there was a significant positive correlation between esterase activity and both the AST/ALT (De Ritis) ratio and C-reactive protein (CRP) levels (Figure 6(a–d)). Although only a few patient samples had an AST/ALT ratio or CRP level in the range of liver or general inflammation, respectively, the trend towards inflammation positively correlated with our esterase activity (Cohen and Kaplan, 1979, Gewurz et al., 1982). Incubating human samples with carboxylesterase inhibitors revealed the same inhibition pattern as our *in vitro* studies and rat plasma suggesting CES1 and CES2 are primary contributors to the observed fluorescent signal (Figure 6(e)). Collectively, our data support the possibility of using endogenous ER resident proteins to monitor changes in ER calcium homeostasis in patients.

Discussion

Proteins with KDEL-like tails have the potential to be used as disease biomarkers. Under normal conditions, proteins with KDEL-like tails are maintained within the ER (Raykhel *et al.*, 2007, Mei et al., 2017). However, there is evidence that some ER luminal proteins with a KDEL sequence are secreted following treatment with calcium ionophores, which suggests that ER resident proteins are sensitive to changes in ER calcium (Booth and Koch, 1989). Recent studies suggest that MANF, an ER resident protein with a KDEL-like C-terminus, may be useful as a biomarker for ER stress in the kidney (Kim et al., 2016) and diabetes (Galli et al., 2016, Wu et al., 2017). Recent work from our laboratory suggests around 80 proteins may be secreted in response to ER calcium depletion (Trychta, Harvey, under

review). In our search for an endogenous marker of ER calcium dysregulation, carboxylesterases were attractive candidates. These enzymes are known to be localized to the ER lumen and have the ability to metabolize esters, thioesters, amides, and carbamates. Multiple carboxylesterase isoforms exist with overlapping substrate specificities for both the activation and inactivation of drugs, toxins, carcinogens, and fatty acid esters. Here we show an increase in extracellular esterase activity following the depletion of ER luminal calcium which supports a model in which carboxylesterases are secreted from the cell following a decrease in luminal ER calcium. Importantly, our *in vitro* experiments demonstrate that increased extracellular esterase levels are not accompanied by decreased cell viability, suggesting that carboxylesterase secretion is not due to cell lysis. While the loss of ER localization and its effects on the endogenous activity of carboxylesterases requires further study, our current results support the use of extracellular carboxylesterase activities to assess ER calcium homeostasis by sampling extracellular fluid, adding a fluorescein diester substrate, and quantifying the fluorescence produced by the selective esterase-ester interaction.

The methods we describe herein focus on measuring fluorescence using a plate reader, however, the original paper describing the fluorescein-CM2 substrate is an imaging-based study (Tian et al., 2012). Although it is theoretically possible that endogenous esterases could be detected *in situ* using the described fluorogenic substrate, complications may arise regarding the membrane permeability of the substrate. Such an approach needs further evaluation and optimization. Further studies may also provide insight into the intracellular and extracellular behavior of endogenous esterases.

We show that esterase activities from at least two carboxylesterases, CES1 and CES2, are elevated in cell culture media and rat plasma following pharmacological depletion of ER calcium. The tissue source of carboxylesterase release is an important issue to consider. Using small molecule inhibitors we show that the fluorescent activity of our substrate is linked to carboxylesterase activity with CES1 and CES2. The highest quantities of CES1 in humans and rats are found in the liver and we hypothesize that our measured activity in plasma is coming from this major tissue source of carboxylesterase (Taketani et al., 2007, Zhang et al., 2002). However, we cannot exclude other tissues as contributing sources of esterase activity. In particular, the small intestine is noted to contain significant levels of CES2 (Taketani et al., 2007, Zhang et al., 2002). Performing tissue selective knockouts of CES could be used to identify specific sources of CES activity.

For the human component of this study, we investigated blood samples from a population of currently drinking alcohol-dependent individuals in order to detect liver and general inflammation markers. Esterase activity positively correlated with clinical measures of liver and general inflammation (AST/ALT ratio and CRP concentration, respectively) in patients. Notably, CRP is a blood marker of general inflammation that is normally retained in the ER by two CRP-binding carboxylesterases that contain the KDEL-like C-terminal sequences HIEL and HTEL (Gewurz et al., 1982, Yue et al., 1996). This, along with the fact that prolonged ER stress is linked to inflammation and has been associated with reduced carboxylesterase steady state levels and reduced CRP binding capacity, makes it expected that CRP levels would track with our endogenous esterase (Zhang and Kaufman, 2008,

Macintyre et al., 1994). Accordingly, during the acute phase inflammatory response, there is reduced binding of these esterases with CRP, resulting in increased CRP secretion into the extracellular environment, which suggests that ER resident protein retention can change depending on pathophysiologic state (Macintyre et al., 1994).

In rat serum, extracellular CRP levels and esterase activity are increased following treatment with Tg (Supplementary Figure 4(a)). However, the kinetics of the two markers differed with esterase levels significantly increased 24 h after Tg and CRP levels showing a delayed increase only reaching significance 48 h post-Tg. Given that CRP is regulated by inflammation and its retention in the ER appears to be connected to other proteins, it may not serve as a direct marker for ER calcium depletion. Importantly, albumin was not increased 48 h post-Tg indicating that the increases seen in esterase and CRP are not due to increased general protein secretion (Supplementary Figure 4(b)). Concurrent monitoring of CRP and esterase levels could be informative in the study, diagnosis, and treatment of diseases associated with ER calcium dysfunction and inflammation. The ability to monitor endogenous esterase activity in the extracellular space could also have therapeutic value in the study of drug biotransformation as circulating carboxylesterase levels are known to affect the hydrolysis of angiotensin-converting enzyme inhibitors, anti-tumor drugs, and drugs of abuse (Geshi et al., 2005, Humerickhouse et al., 2000, Kamendulis et al., 1996).

While the high inter-individual variability of carboxylesterase expression may preclude these proteins from being obvious biomarkers (Yang et al., 2009), the possibility warrants further study. Although variability may cause complications when comparisons are made between patients, the newly discovered ability to track ER calcium depletion in a single patient over time would still have a significant impact. Monitoring esterase activity in extracellular fluids, has the potential for delineating the cause and effect relationship between ER calcium dysfunction and disease pathogenesis. For example, we recently showed that esterase activity increases in the plasma of rats fed a high fat diet and correlates with GLuc-ASARTDL secretion from the liver in an ER calcium-dependent manner (Wires et al., 2017).

Conclusion

While the introduction of transgenes or chemical dyes to measure ER calcium levels may be more sensitive to perturbations in calcium at the cellular level, we have described a blood-based biomarker of changes in ER calcium homeostasis. Our data support a model in which ER calcium depletion triggers the secretion of ER resident carboxylesterases which can be detected by sampling extracellular fluids, e.g. cell culture media or plasma. Further evaluation of this approach in other pathological states where ER calcium depletion has been implicated or identified as a contributing mechanism will aid in monitoring, understanding, and treating human diseases

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Emily Simons Wires for technical assistance. This work was supported by the Intramural Research Programs at the National Institute on Drug Abuse (KR, LL, and BKH) and the National Institute on Alcohol Abuse and Alcoholism (KR and LL).

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Clinical significance

- Esterases are present in plasma and serum and can be measured using a fluorescent assay.
- Increased extracellular esterase activity corresponds to depletion of luminal ER calcium.
- Monitoring esterase secretion may provide insight into the role of ER calcium in disease.

PLE - KIKHAEL **CES1 - QTEHIEL** **CES2 - EERHTEL**

Figure 1: KDEL-like carboxy-terminal tails of PLE, CES1 and CES2.
Primary structure of the terminal seven amino acids of PLE, CES1, and CES2

Figure 2: PLE can be detected using a fluorescein diester substrate.

(a) Fluorescence observed in unconditioned esterase assay medium and medium collected from SH-SY5Y cells transiently transfected with a control or PLE vector (mean $\pm$ s.d., $n=3$). (b) Extracellular fluorescence of media from SH-SY5Y cells transiently transfected with PLE following the addition of 100 μ M of fluorescein-CM2 substrate at various pH to medium at 45°C (mean $\pm$ s.d., $n=4$, one-way ANOVA with Tukey's multiple comparisons test, $*p<0.05$, $**p<0.01$). (c) Extracellular fluorescence from SH-SY5Y cells transiently transfected with PLE following the addition of 100 μ M of pH 5.0 fluorescein-CM2 substrate to medium at various temperatures (mean $\pm$ s.d., $n=4$, one-way ANOVA for pH 3–8.5 with Tukey's multiple comparisons test, $*p<0.05$, $***p<0.001$, $****p<0.0001$). (d) Extracellular fluorescence from SH-SY5Y cells transiently transfected with PLE following the addition of various concentrations of pH 5.0 fluorescein-CM2 substrate to medium at 45°C (mean $\pm$ s.d., $n=4$, one-way ANOVA with Tukey's multiple comparisons test, $*p<0.05$). (e) Fluorescence of 100 μ L sample containing various volumes of medium from SH-SY5Y cells transiently transfected with PLE (mean $\pm$ s.d., $n=4$). (f) Extracellular fluorescence from SH-SY5Y cells transiently transfected with PLE and incubated for 30 min with carboxylesterase inhibitors (mean $\pm$ s.d., $n=3$). (g) Fluorescence in medium following 30 min incubation with carboxylesterase inhibitors (mean $\pm$ s.d., $n=3$).

Figure 3: Endogenous esterase activity can be monitored in three different cell lines.

(a) Tg (200nM) and vehicle induced secretion of GLuc-ASARTDL and endogenous esterase in SH-SY5Y cells stably expressing GLuc-ASARTDL over the course of 8 h (mean $\pm$ SEM, n=24, 3 independent experiments, two-way ANOVA with Dunnett's multiple comparisons test, ****p<0.0001 esterase fluorescence vs. 0 h, ##p<0.01 GLuc luminescence versus 0 h, #####p<0.0001 GLuc luminescence versus 0 h). (b) Fluorescent esterase activity in Hep3B and HepG2 cell culture media following Tg-induced ER calcium depletion with 200 nM Tg for 8 h (mean $\pm$ SEM, n=6, unpaired two-tailed t-test, **p<0.01, ****p<0.0001). (c) Fluorescence of medium from Hep3B cells treated with 200nM Tg for 8 h following a 30 min incubation with carboxylesterase inhibitors (mean $\pm$ s.d., n=3).

Figure 4: Esterase activity increases following ER calcium depletion.

Fluorescent esterase activity in Hep3B culture medium 8 h after 200 nM Tg or 3 μg/mL tunicamycin treatment (mean ± SEM, n=16, 2 independent experiments, one-way ANOVA with Dunn's multiple comparisons test to vehicle, ***p<0.001). (b) Fluorescent esterase activity in Hep3B cell culture medium following Tg-induced ER calcium depletion (mean ± SEM, n=8, one-way ANOVA with Holm-Sidak's multiple comparisons to vehicle, ****p<0.0001). (c) Fluorescent esterase activity in medium from Hep3B cells after a 2 h CPA treatment followed by a washout and 8 h incubation (mean ± SEM, n=4, one-way ANOVA with Dunnett's multiple comparisons test to vehicle, *p<0.05, ***p<0.001, ****p<0.0001). (d) Fluorescent esterase activity in medium from Hep3B cells 8 h after CPA treatment (10 μM) with or without a 30 μM dantrolene pre-treatment (mean ± SEM, n=14, 3 independent experiments, two-way ANOVA with Bonferroni's multiple comparisons test, p<0.001 for vehicle versus CPA, ****p<0.0001 for vehicle versus dantrolene). (e) OGD and normoxia induced extracellular esterase activity in SH-SY5Y cells exposed to 16 h of OGD followed by 8 h of normoxia (mean ± SEM, n=16, 2 independent experiments, two-way ANOVA with Sidak's multiple comparisons test, p<0.0001 for overall OGD effect, ****p<0.0001 for normoxia versus OGD). (f) OGD and normoxia induced extracellular esterase activity in SH-SY5Y cells exposed to 16 h of OGD with or without 30 μM dantrolene followed by 8 h of normoxia (mean ± SEM, n=24, 3 independent experiments, two-way ANOVA with Dunnett's multiple comparisons test, p<0.0001 for overall OGD effect, ****p<0.0001 for OGD vehicle versus dantrolene).

Figure 5: Esterase activity measured in rat plasma increases following Tg treatment.

(a) Tg (1 mg/kg, i.p.) and vehicle (i.p.) induced secretion of GLuc-ASARTDL and endogenous esterase in rat plasma relative to day 14 pre-Tg levels (mean $\pm$ SEM, $n=3$ rats, one-way ANOVA for GLuc-ASARTDL and esterase with Dunnett's multiple comparisons test to day 14 pre-Tg, $*p<0.05$, $**p<0.01$). (b) Fluorescence of rat plasma treated with esterase substrate following a 30 min incubation with carboxylesterase inhibitors (mean $\pm$ s.d., $n=3$).

Figure 6: Esterase activity can be monitored in human samples.

Correlations of esterase activity in human blood with (a) age (n=39 participants), (b) BMI (n=39 participants), (c) AST/ALT ratio (n=38 participants), and (d) C-reactive protein concentrations (n=33 participants). (e) Fluorescence in human plasma following 30 min incubation with carboxylesterase inhibitors (mean $\pm$ s.d., n=3).

Table 1.

Nucleotide sequences for primers and probes used in real time RT-qPCR. Probes for markers of ER stress signaling activation were labelled with FAM/BHQ1 and probes for reference genes with HEX/BHQ1.

Target	Forward	Reverse	Probe
Human ASNS (NM_001178077)	ggattggctgcctttatcagg	ggcttcttcagctgcttcaac	tggactccagcttggtgctgcc
Human BiP (NM_005347)	gttggtggccactaatggagatac	ggagttctgcacagctctattg	acgtggtcaaagtcttccacca
Human ERdj4 (NM_012328)	gccatgaagtaccacctg	ccactagtaaaagcactgtgtc	ctgcaatctctgaatttgcctcagc
Human Ube2i (NM_003345)	gtgtgcctgtccatcttagag	gctgggtcttgatatttggtc	caaggactggaggccagccatcac

ASNS, asparagine synthetase; BiP, binding immunoglobulin protein; ERdj4, endoplasmic reticulum DnaJ 4; Ube2i, ubiquitin-conjugating enzyme E2I