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Calmodulin Binding to Death Receptor 5-mediated Death-inducing Signaling Complex in Breast Cancer Cells

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Abstract

Activation of death receptor-5 (DR5) leads to the formation of death inducing signaling complex (DISC) for apoptotic signaling. TRA-8, a DR5 specific agonistic antibody, has demonstrated significant cytotoxic activity *in vitro* and *in vivo* without inducing hepatotoxicity. Calmodulin (CaM) that is overexpressed in breast cancer plays a critical role in regulating DR5-mediated apoptosis. However, the mechanism of CaM in regulating DR5-mediated apoptotic signaling remains unknown. In this study, we characterized CaM binding to DR5-mediated DISC for apoptosis in TRA-8 sensitive breast cancer cell lines using co-immunoprecipitation, fluorescence microscopic imaging, caspase signaling analysis and cell viability assay. Results show that upon DR5 activation, CaM was recruited into DR5-mediated DISC in a calcium dependent manner. CaM antagonist, trifluoperazine (TFP), inhibited CaM recruitment into the DISC and attenuated DISC formation. DR5 oligomerization is critical for DISC formation for apoptosis. TFP decreased TRA-8 activated DR5 oligomerization, which was consistent with TFP's effect on DR5-mediated DISC formation. TFP and Ca²⁺ chelator, EGTA, impeded TRA-8 activated caspase-dependent apoptotic signaling, and TFP decreased TRA-8 induced cell cytotoxicity. These results demonstrated CaM binding to DR5-mediated DISC in a calcium dependent manner and may identify CaM as a key regulator of DR5-mediated DISC formation for apoptosis in breast cancer.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article except that Dr. Tong Zhou and Dr. Donald J. Buchsbaum have intellectual property right in TRA-8.

AUTHOR CONTRIBUTIONS

R. M. F., Y. S. and T. Z. designed the research. R. M. F. conducted the biochemical and fluorescence microscope imaging experiments and H. K. performed fluorescence microscope imaging analysis. K. R. Z. provided guidance for fluorescence microscope imaging experiments and D. J. B provided guidance for biochemical experiments. R. M. F., H. K. and Y. S. wrote the manuscript. K. R. Z. and D. J. B provide inputs and assistance for the manuscript writing. All authors reviewed the results and approved the final version of the manuscript.

Keywords

Calmodulin; death receptor 5; DISC; DR5 oligomerization; apoptosis; breast cancer cells

Breast cancer is the second most common malignancy among American women and is the second leading cause of cancer death in the United States (DeSantis et al., 2011). Classification of breast cancers into receptor-based subtypes, including estrogen receptor (ER) positive or triple-negative breast cancers, establish targets that guide individualized treatment for patients (Sorlie, 2009). Drug resistant ER-positive breast cancers and triple-negative breast cancers are prolific breast cancers with poor clinical outcome (Guarneri and Conte, 2004). Better understanding of the pathogenesis of breast cancer could facilitate the development of novel strategies for targeted therapy for effective breast cancer treatment. Death receptor 5 (DR5) is a member of the tumor necrosis factor (TNF) receptor superfamily and is one of five receptors that bind to TRAIL (TNF-receptor apoptosis inducing ligand) (Ashkenazi et al., 1999; Pitti et al., 1996; Walczak et al., 1999). DR5 is expressed and upregulated in most breast cancer cells (Buchsbau et al., 2003; Dong et al., 2008; McCarthy et al., 2005). TRA-8 is a DR5 specific agonistic antibody and has demonstrated tumoricidal activity without inducing hepatotoxicity (Ichikawa et al., 2001). DR5 contains a cytoplasmic death domain (Pitti et al., 1996) and transduces its apoptotic signal via forming the death inducing signaling complex (DISC) and activation of caspase signaling (Sprick et al., 2000). DR5 death domain (DR5 DD) mutants that change DR5 DD conformation, dynamical motions and the interactions between DR5 DD and Fas associated death domain protein (FADD) have been suggested to prevent the proper assembly of DISC (Bin et al., 2007; McDonald et al., 2001; Yang and Song, 2016). Upon DR5 activation by TRA-8 binding to its extracellular domain, DR5 oligomerizes into homotrimers (Hymowitz et al., 1999). TRA-8 activated DR5 aggregation is essential to drive intracellular signals for cell death (Dumitru and Gulbins, 2006; Wagner et al., 2007), and the degree of DR5 oligomerization is directly related to the TRA-8 driven cytotoxicity in breast cancer (Kim et al., 2016). DR5 binds to FADD, then FADD recruits caspase-8 to form the DISC to initiate DR5 mediated apoptotic signaling pathway (Daniel et al., 2001; Kischkel et al., 1995; Kischkel et al., 2000; Sprick et al., 2000). TRA-8 activation of DR5 can induce apoptosis via a caspase-dependent mechanism in human breast cancer cell lines (Rahman et al., 2009).

Calmodulin (CaM) functions as an intracellular mediator of Ca^{2+} signals and regulates various cellular processes (Cohen, 1988). Breast tumor malignancy is associated with increased CaM expression (Hait and Lazo, 1986; Wei et al., 1982). CaM antagonist regulation of DR5 mediated apoptotic signaling has been reported in human lung cancer cells and hepatocellular carcinoma cells (Hwang et al., 2009; Wang et al., 2009). However, the mechanisms for the role of CaM in DR5-mediated apoptotic signaling remain unknown. Our recent study shows that CaM directly bind to DR5 in a Ca^{2+} dependent manner in breast cancer cells (Fancy et al., 2016b), which lays the basis for this study of the CaM binding to DR5-mediated DISC to signal apoptosis in breast cancer cells.

In this study, we characterized Ca^{2+} -dependent CaM binding to DR5-mediated DISC for apoptosis in breast cancer cells. ER-positive (ZR-75-1) and triple-negative (MDA-MB-231)

breast cancer cell lines used for this study are sensitive to TRA-8 (Buchsbaum et al., 2003; Oliver et al., 2012). Trifluoperazine (TFP) is a potent antipsychotic phenothiazine drug and has been commonly used in studies for its inactivation of CaM (Klevit et al., 1981; Levin and Weiss, 1977; Massom et al., 1990; Massom et al., 1991). TFP binding to CaM induced CaM conformational changes, which could further affect CaM binding to other proteins for its functional activities (Klevit et al., 1981; Pan et al., 2011a; Vandonselaar et al., 1994). We determined the effect of TFP on CaM recruitment into DISC, DR5-mediated DISC formation, TRA-8 activated DR5 oligomerization, and TRA-8 activated caspase-dependent apoptotic signaling and apoptosis in ZR-75-1 and MDA-MB-231 breast cancer cells.

EXPERIMENTAL PROCEDURES

Cell Culture

ZR-75-1 and MDA-MB-231 breast cancer cells were given by Dr. Donald Buchsbaum and Dr. Tong Zhou at the University of Alabama at Birmingham (UAB). ZR-75-1 cells were cultured in HAM's K-12 media (Hyclone GE Healthcare Lifesciences, South Logan, UT) with 20% FBS. MDA-MB-231 cells were cultured in DMEM media (Thermo Fisher Scientific, Waltham, MA) with 10% FBS and supplemented with MEM vitamins, nonessential amino acids, sodium pyruvate and L-glutamine. Both cell lines were maintained in the medium with 10–20% FBS, supplemented with 1% penicillin, 1% streptomycin and 1% amphotericin B antibiotic at 37°C, 5% CO₂ and 95% relative humidity atmosphere.

Protein Complex Immunoprecipitation

To assess CaM recruitment into DR5-mediated DISC, MDA-MB-231 or ZR-75-1 cells were untreated, treated with 1 µg/mL of TRA-8 (Daiichi-Sankyo Company, Ltd, Tokyo, Japan) for 3 hours or 500 ng/mL recombinant soluble human TRAIL (Enzo Life Sciences Inc., Farmingdale, NY) for 24 hours. Following the treatment, MDA-MB-231 or ZR-75-1 cells were lysed using the buffer with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1:100 Halt protease inhibitor (Thermo Fisher Scientific, Waltham, MA) at 4°C for 30 minutes. The total protein concentration was determined using bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA) and a total protein concentration of 5 mg/mL was used for co-immunoprecipitation (co-IP) experiments. MDA-MB-231 and ZR-75-1 cell lysates were precleared with 40 µL of 4B-sepharose beads (Sigma-Aldrich, St. Louis MO) at 4 °C for 1 hour. DR5-mediated DISC was co-IP from MDA-MB-231 or ZR-75-1 cell lysates using anti-DR5 (D-6) antibody (Santa Cruz Biotechnology, Inc., Santa Barbara, CA, specificity: DR5, source: mouse, Cat. sc-166624, Lot# C2811) incubated overnight at 4 °C in cell lysates. In the following morning, cell lysates were incubated with 40 µL of protein A/G PLUS-Agarose beads for 2 hours. As control experiments, MDA-MB-231 or ZR-75-1 cell lysates were incubated with mouse iso-IgG antibody conjugated sepharose beads (Cell Signaling Technology, Beverly, MA, specificity: none, source: mouse, Cat. 3420S, Lot# 5) or only 4B-sepharose beads (Sigma-Aldrich, St. Louis MO) overnight at 4 °C.

To determine the calcium dependence of DR5-mediated DISC formation, MDA-MB-231 or ZR-75-1 cells were treated with 1 µg/mL of TRA-8 alone for 3 hours, or were pre-treated with 2 mM of Ca²⁺ chelator, EGTA, for 30 minutes and then TRA-8 (1 µg/mL) for 3 hours. The effect of CaM antagonist, TFP, on DR5-mediated DISC was determined by preincubating MDA-MB-231 or ZR-75-1 cells with 10 µM of TFP (MP Biomedicals, Solon, OH) for 15 or 30 minutes followed by 1 µg/mL of TRA-8 treatment for 3 hours. Following the treatments MDA-MB-231 or ZR-75-1 cells were lysed using the buffer with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1:100 Halt protease inhibitor at 4°C for 30 minutes. The total protein concentration was determined using the BCA assay and a total protein concentration of 5 mg/mL was used for co-IP experiments. MDA-MB-231 and ZR-75-1 cell lysates were precleared with 40 µL of 4B-sepharose beads, then DR5-mediated DISC was co-IP from MDA-MB-231 or ZR-75-1 cell lysates using anti-DR5 (D-6) antibody (Santa Cruz Biotechnology, Inc., Santa Barbra, CA, specificity: DR5 source: mouse, Cat. sc-166624, Lot# C2811) with protein A/G Plus-Agarose beads (Santa Cruz biotechnology, Inc., Santa Barbra, CA).

Following IP experiments, antibodies or beads used for IP were washed using 50 mM Tris-HCL pH 7.5, 150 mM NaCl, 0.5% Triton X-100 wash buffer five times. After washing, 40 µL of 2X laemmli sample buffer with 2-mercaptoethanol were added to IP samples. IP and lysate samples in laemmli sample buffer were heated at 100 °C for 5 minutes and then centrifuged for 1 minute at 3000 rpm. 40 µL of IP sample and 35–40 µg of total protein from lysate were loaded into a SDS-PAGE gel for western blotting.

Western Blot Analysis

CaM binding to DR5-mediated DISC and the effect of CaM antagonist, TFP, on DR5-mediated caspase activation were assessed by western blot analyses. Samples from IPs or cell lysates in laemmli buffer were heated at 100 °C for 5 minutes. Protein samples were resolved by SDS-PAGE and transferred onto a 0.45 µm nitrocellulose membrane (Bio-Rad, Hercules, CA). For the samples for co-IPs of DR-mediated DISC, membranes were incubated overnight at 4°C with primary antibodies against DR5 (Abcam Cambridge, MA, specificity: DR5 isoform 1 and 2, source: rabbit, Cat. Ab47179, Lot# GR199335-1), FADD (Cell Signaling Technologies Beverly, MA, specificity: FADD, source: rabbit, Cat. 2782S, Lot# 4), Caspase-8 (Cell Signaling Technologies Beverly, MA, specificity: Caspase-8 (D3562) (57/10kDa), source: rabbit, Cat. 2782S, Lot# 2), or CaM (Millipore Billerica, MA, source mouse, Cat. 05173, Lot# 2123697). For cell lysate samples for caspase signaling analysis, membranes were incubated overnight at 4°C with primary antibodies against Caspase-8 (Cell Signaling Technologies Beverly, MA, specificity: Caspase-8 (D3562) (57/10kDa), source: rabbit, Cat. 4790S, Lot# 2), Caspase-9 (Cell Signaling Technology, Beverly, MA, specificity: Caspase-9 (35/37/47kDa), source: rabbit, Cat. 9502S, Lot#8), Caspase-3 (Cell Signaling Technology, Beverly, MA, specificity: Caspase-3 (35/19/17kDa), source: rabbit, Cat. 9662S, Lot# 15), and PARP (Cell Signaling Technology, Beverly, MA, specificity: PARP1(116/89 kDa), source: rabbit, Cat. 9542S, Lot# 13). Following overnight antibody incubation, membranes were washed three times with TBST, and then incubated with horseradish peroxidase-conjugated secondary antibodies (Southern Biotechnologies Birmingham, Alabama, specificity: anti-rabbit IgG (H+L) source: goat Cat. 405-05, Lot#

K0008-VJ213 or specificity: anti-mouse IgG (H+L), source: goat, Cat. 1031-05, Lot# H2710-NA02) at room temperature for 1–2 hour. Blots were developed using enhanced chemiluminescence reagents (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

Fluorescence Microscope Imaging and Analysis

To determine the effect of CaM antagonist, TFP, on TRA-8 activated DR5 oligomerization on MDA-MB-231 and ZR-75-1 cells, we performed fluorescence microscope imaging as introduced in our previous study (Kim et al., 2016). In brief, 1×10^6 of MDA-MB-231 or ZR-75-1 cells were seeded in 35-mm glass bottom culture dishes (MatTek Corp., Ashland, MA) and incubated in the complete medium, as described in the cell culture section, overnight. The monofunctional dye Cy5.5 was conjugated to TRA-8 at a 3:1 molar ratio per manufacturer's instruction. MDA-MB-231 or ZR-75-1 cells were washed with PBS and then treated with 5 μ g/mL of Cy5.5 labeled TRA-8 with or without 10 μ M of TFP for 24 hours. Following treatment, MDA-MB-231 or ZR-75-1 cells were washed with PBS. Nine images at 100X magnification from randomly selected areas were taken per each treatment group using a Leica DMIRE2 inverted microscope equipped with a Nuance camera (CRI Inc., Woburn, MA). The distribution of Cy5.5 labeled TRA-8 was analyzed using a fully automated computer software package as described in our previous study (Kim et al., 2015). In brief, the computer software found the best fitting 2-dimensional Gaussian curve to the distribution of Cy5.5-TRA-8 in each region of oligomer, and the peak amplitude and the full-width at half maximum (FWHM) of the Gaussian curve were extracted. About 100 oligomers were detected in each image (about 900 oligomers per group). The computer software package was developed by Dr. Harrison Kim at UAB using Labview, version 2014 (National Instrument, Austin, TX).

Caspase Signaling Analysis

Effect of CaM antagonist, TFP, and Ca^{2+} chelator, EGTA, on DR5-mediated caspase cleavage was characterized. 4×10^6 - 5×10^6 MDA-MB-231 and ZR-75-1 cells were seeded on 100 mm dishes and cultured in media overnight. The cells were untreated (served as control) or treated with TRA-8 only for 3 hours, treated with TFP or EGTA only, or treated with TFP or EGTA for 30 minutes followed by TRA-8 treatment for 3 hours. Following each treatment, cells were washed with cold PBS and lysed with RIPA buffer with 1:100 Halt protease inhibitor (Thermo Fisher Scientific, Waltham, MA). Samples were sonicated on ice for 10 seconds, and total protein concentration was determined using a BCA assay (Thermo Fisher Scientific, Waltham, MA). Then 6X laemmli buffer with 2-mercaptoethanol was added to samples, 40–50 μ g of total protein were loaded for western blot.

Cell Viability Assay using ATPLite

MDA-MB-231 or ZR-75-1 cells were seeded onto tissue-culture treated 96-well plates (Costar #3595, Corning, Inc., NY; n=3000 per well) in 100 μ L of complete media, as described in the cell culture section, and incubated overnight at 37°C. TFP and TRA-8 were diluted in culture medium from stock solutions immediately before use. Cells were treated with 0.5 μ g/mL of TRA-8 for 3 hours, or 10 μ M of TFP only for 30 minutes or 10 μ M of TFP for 30 minutes followed by 0.5 μ g/mL of TRA-8 for 3 hours, which were the same

treatment conditions as those for the characterization of the effect of TFP on DR5-mediated caspase cleavage as described above. Cell viability was assessed by the measurement of cellular ATP levels using the ATPLite luminescence-based assay (Perkin Elmer Waltham, MA), following the manufacturer's recommended protocol. Cell viability assay was repeated six times.

Statistical Analysis

One-way ANOVA was used to compare the amplitudes of best fitting Gaussian curves for the signal intensities of Cy5.5-TRA-8 on MDA-MB-231 cells or ZR-75-1 cells with/without TFP treatment. Pairwise t-tests were performed on cell viability data. *P*-values less than 0.05 were considered significant. Data are presented as the mean $\pm$ standard error

RESULTS

CaM was recruited into DR5-mediated DISC in a Ca^{2+} -dependent manner in MDA-MB-231 and ZR-75-1 cells

CaM recruitment into DR5-mediated DISC was determined using co-immunoprecipitation (co-IP) experiments. DR5, FADD and Caspase-8 formed the DISC, and CaM was recruited into the DISC upon activation of DR5 by either TRA-8 or TRAIL in MDA-MB-231 cells or ZR-75-1 cells (Fig. 1 and Fig. S1). For co-IP control experiments, DR5-mediated DISC components did not non-specifically bind to the mouse isoIgG-sepharose beads or 4B-sepharose beads in MDA-MB-231 cells or ZR-75-1 cells (Fig. 1 and Fig. S1). In addition, CaM recruitment into DR5-mediated DISC in a Ca^{2+} -dependent manner was characterized using co-IP experiments. For the cells treated with TRA-8 alone or those pretreated with Ca^{2+} chelator (EGTA) followed by TRA-8 treatment, CaM, DR5, FADD and caspase-8 expressions in the cell lysate before co-IP were not affected by EGTA treatment in either MDA-MB-231 (Fig. 2A) or ZR-75-1 cells (Fig. 2B). However, with EGTA treatment, TRA-8 activated DR5 recruitment of FADD, caspase-8, and CaM to form DR5-mediated DISC was significantly decreased in either MDA-MB-231 cells (Fig. 2A) or ZR-75-1 cells (Fig. 2B). These results confirm that CaM was recruited into DR5-mediated DISC in a calcium dependent manner.

CaM antagonist, TFP, attenuated DR5-mediated DISC formation in MDA-MB-231 and ZR-75-1 cells

To further verify CaM recruitment into DR5-mediated DISC, we investigated the effect of TFP on DR5-mediated DISC formation and on the recruitment of CaM into the DISC. CaM, DR5, FADD and caspase-8 expressions in the cell lysate before co-IP were not affected with or without TFP in either MDA-MB-231 (Fig. 3A) or ZR-75-1 cells (Fig. 3B). With TRA-8 activation of DR5 and using anti-DR5 (D-6) antibody with protein A/G Plus-Agarose beads for co-IP experiments, co-IP experiments show that TFP inhibited DR5-mediated DISC formation and TFP also decreased CaM recruitment into DR5-mediated DISC in both cell lines. For MDA-MB-231 cells, 30 minutes of TFP pretreatment resulted in a higher degree of inhibition of CaM recruitment into DR5-mediated DISC than that of 15 minutes of TFP treatment (Fig. 3A). These results confirmed CaM involvement in DR5-mediated DISC formation.

TFP decreased TRA-8 activated DR5 oligomerization in MDA-MB-231 and ZR-75-1 cells

TRA-8 activated DR5 oligomerization is critical for DISC formation to signal apoptosis (Festjens et al., 2006). We characterized the effect of CaM antagonist, TFP, on TRA-8 activated DR5 oligomerization to further understand the role of CaM in DR5-mediated DISC formation for apoptosis. Figure 4A shows the representative images of Cy5.5 labeled TRA-8 on MDA-MB-231 or ZR-75-1 cells with or without TFP treatment, in which the same color scale was applied for all images. The fluorescence images were overlapped with bright field images, showing the cell morphology. Cy5.5 labeled TRA-8/DR5 oligomerization was observed in both MDA-MB-231 and ZR-75-1 cells (Fig. 4A). Figure 4B shows the Gaussian curves fitting to the distribution of Cy5.5-TRA-8 on the representative cells indicated with white arrows in Fig. 4A. Cy5.5-TRA-8 distribution followed a Gaussian curve, and TFP decreased the peak signal intensity of Cy5.5-TRA-8 in either MDA-MB-231 or ZR-75-1 cells (Fig. 4B). Figures 4C and 4D show the peak amplitudes of TRA-8/DR5 oligomers when cells were treated with Cy5.5-TRA-8 alone or in combination with TFP in MDA-MB-231 and ZR-71-1 cell lines, respectively. The mean peak amplitudes after TFP treatment were 4126 ± 71 and 2948 ± 48 in MDA-MB-231 and ZR-71-1 cell lines, respectively, which were lower than those before TFP treatment (5354 ± 106 and 5096 ± 68) with one-way ANOVA statistical significance ($p < 0.05$ in both cell lines). The results demonstrated that TFP inhibited DR5 aggregation in both MDA-MB-231 and ZR-71-1 cell lines, which will directly attenuate DR5-mediated DISC formation as shown in Fig. 3.

TFP and EGTA inhibited DR5-mediated caspase signaling in both MDA-MB-231 and ZR-75-1 cell lines

DISC causes the autoproteolytic cleavage and activation of caspase-8, initiating one of two apoptotic pathways converging at the activation of caspase-3 and PARP (Kischkel et al., 2000). As shown in Figs. 1–3, upon TRA-8 activation of DR5, DR5 recruited FADD and caspase-8 to form DISC and Ca^{2+} chelator, EGTA, and CaM antagonist, TFP, inhibited DR5-mediated DISC formation. Accordingly, we determined the DR5 activated DISC-induced cleavage of caspase-8, caspase-3, and PARP and the effect of TFP and EGTA on DR5-mediated caspase signaling for apoptosis. Results showed that TRA-8 induced caspase-8, caspase-3, and PARP cleavage, but not caspase-9 cleavage for either MDA-MB-231 (Fig. 5A) or ZR-75-1 cell line (Fig. 5B). However, when the cells were pretreated with TFP or EGTA, TRA-8 activated caspase-8, caspase-3, and PARP cleavages were reduced in either MDA-MB-231 (Fig. 5A) or ZR-75-1 cells (Fig. 5B).

TFP decreased TRA-8 induced cytotoxicity in both MDA-MB-231 and ZR-75-1 cell lines

We also determined the effect of TFP on TRA-8 induced cytotoxicity in MDA-MB-231 and ZR-75-1 cells using ATPLite cell viability assay. With TFP treatment alone, cells remain cell viability at least 90% for either MDA-MB-231 or ZR-75-1 cell line (Fig. 6). TRA-8 treatment alone reduced the cell viability to 29% for MDA-MB-231 (Fig. 6A) and 31% for ZR-75-1 cells (Fig. 6B). Combined TRA-8 and TFP treatment resulted in the cell viability of 45% for MDA-MB-231 (Fig. 6A) and 51% for ZR-75-1 cells (Fig. 6B). The results

demonstrated that TFP significantly reduced TRA-8 induced cytotoxicity in both MDA-MB-231 (Fig. 6A) and ZR-75-1 (Fig. 6B) cells (pairwise t-test, $p < 0.05$).

DISCUSSION

We recently demonstrated the Ca^{2+} -dependent binding between CaM and DR5 in MDA-MB-231 and ZR-75-1 breast cancer cells (Fancy et al., 2016a). To understand the biological significance of CaM-DR5 interactions, we characterized CaM recruitment into DR5-mediated DISC in a Ca^{2+} -dependent manner and the effect of CaM antagonist, TFP, on DR5-mediated DISC formation and apoptotic signaling in MDA-MB-231 and ZR-75-1 breast cancer cells in this study.

DISC formation is a critical step in DR5-mediated signaling of apoptosis (Daniel et al., 2001; Kischkel et al., 2000; Sprick et al., 2000; Wang and El-Deiry, 2003). Co-immunoprecipitation results showed that upon DR5 activation by either TRA-8 or TRAIL, CaM was recruited into the DR5-mediated DISC (Fig. 1 and Fig. S1). The Ca^{2+} chelator EGTA and CaM antagonist TFP inhibited CaM recruitment into DR5-mediated DISC and attenuated DR5-mediated DISC formation in both MDA-MB-231 and ZR-75-1 cells (Figs. 2 and 3). The results demonstrated that CaM recruitment into DR5-mediated DISC was calcium dependent. TFP molecules bound to CaM could change CaM conformation (Pan et al., 2011b), which could affect CaM-DR5 binding, further affect DR5 recruitment of FADD for DISC formation as observed in Fig. 3. The results provide the biological significance for CaM-DR5 binding observed in our previous study (Fancy et al., 2016b), and present the potential molecular mechanism for the role of CaM in DR5-mediated apoptotic signaling in breast cancer.

TRA-8 activated DR5 aggregation was essential to drive intracellular signals for death (Dumitru and Gulbins, 2006; Wagner et al., 2007). Fluorescence microscopic imaging of TRA-8-activated DR5 oligomers has been able to predict TRA-8 therapeutic efficacy in breast cancer mouse model (Kim et al., 2016). Results of fluorescence microscopic imaging analysis of TRA-8 activated DR5 oligomerization in this study showed that CaM antagonist, TFP inhibited DR5 oligomerization in MDA-MB-231 and ZR-75-1 cells (Fig. 4), indicating TFP could disrupt DR5 oligomerization and thus impede cytotoxicity of TRA-8 in TRA-8 sensitive MDA-MB-231 or ZR-75-1 cells. DR5 conformation could be changed by the changed CaM-DR5 interactions by TFP, further affecting DR5 oligomerization. Oligomerization of DISC's constituents is critical for DISC activation for apoptosis (Festjens et al., 2006). The effect of TFP on DR5 oligomerization (Fig. 4) and DISC formation (Fig. 3) may result from TFP attenuating CaM-DR5 binding, changing DR5 structure and conformation, further affecting DR5 oligomerization and DR5 recruiting FADD for DISC formation. The DR5 oligomerization has been proved to be directly related to TRA-8 induced apoptosis in breast cancer (Kim et al., 2016).

Kinases such as Src and casein kinase 1 are known to phosphorylate caspase-8 and FADD respectively, and the phosphorylation of DISC components of FADD and caspase-8 has been shown to impede DISC formation and death receptor induced apoptosis (Cursi et al., 2006; Izeradjene et al., 2004; Wang et al., 2013). Studies have shown that CaM directly binds Src

and CaM and Src are recruited into death receptor induced DISC (Yuan et al., 2011; Yuan et al., 2015). Studies also show that the death domain-containing receptors of the tumor necrosis factor (TNF) family, including DR5, contains a conserved phosphotyrosine-containing motif and this motif could bind to the Src homology domain 2 (SH2)-containing phosphatases, the inhibitory phosphatases, including tyrosine phosphatase-1 (SHP-1) and SHP-2 (Daigle et al., 2002). These studies suggest that CaM-mediated Src recruitment into DR5-mediated DISC could cause DR5 phosphorylation in initial stages of activation, however, the binding of DR5 with SHP-1 could cause DR5 de-phosphorylation and facilitate DR5 clustering to form DR5-mediated DISC for apoptosis in TRA-8 sensitive MDA-MB-231 and ZR-75-1 breast cancer cell lines. It suggests that the role of CaM in DR5 oligomerization and DR5-mediated DISC formation in breast cancer cells (Fig. 1–4) as observed in this study could be directly related to Src and SHP-1 regulation of the phosphorylation of DR5 and caspase -8, the key components of DISC.

The results of TFP inhibition of DR5 oligomerization provide the molecular insight for the effect of TFP on DR5-mediated DISC formation in MDA-MB-231 and ZR-75-1 cells and offer an important perspective of quantifying the DR5 oligomerization as a potential biomarker to investigate TRA-8/TRAIL treatment in breast cancer.

TRA-8 activated DR5-mediated DISC formation initiates DR5 mediated apoptotic signaling via caspase cleavage and activation (Amm et al., 2011; Buchsbaum et al., 2003; Li et al., 2006; Oliver et al., 2012). Our caspase signaling analysis results showed that CaM antagonist, TFP, and Ca^{2+} chelator, EGTA decreased TRA-8 activated caspase-8, caspase-3, and PARP cleavage for apoptosis in MDA-MB-231 and ZR-75-1 breast cancer cells (Fig. 5). The cell viability assay results further confirmed that TFP significantly decreased TRA-8 induced cytotoxicity in both MDA-MB-231 and ZR-75-1 cell lines (Fig. 6). The cell viability assay results were consistent with the co-immunoprecipitation results that TFP inhibited DR5-mediated DISC formation (Fig. 3), fluorescence microscope imaging results that TFP decreased TRA-8 activated DR5 oligomerization (Fig. 4), and caspase signaling analyses that TFP decreased TRA-8 activated caspase-8, caspase-3, and PARP cleavage for apoptosis (Fig. 5) in MDA-MB-231 and ZR-75-1 breast cancer cells.

Previous studies have shown that not only mediating apoptosis, DR5 also mediates anti-apoptotic signaling in breast cancer cells (Sun et al., 2008). CaM directly binds to DR5 in breast cancer cells (Fancy et al., 2016b). The effect of TFP on cell viability in MDA-MB-231 and ZR-75-1 breast cancer cells as shown in Fig. 6 could not only result from TFP effect on DR5-mediated DISC formation, could be also related to TFP effect on DR5-mediated anti-apoptotic signaling. ZR-75-1 and MDA-MB-231 breast cancer cell lines used in this study are sensitive to TRA-8 (Buchsbaum et al., 2003; Oliver et al., 2012). There are also breast cancer cell lines that are resistant to TRA-8 (Buchsbaum et al., 2003; Oliver et al., 2012). Effect of TFP on DR5 oligomerization and DR5-mediated DISC formation in TRA-8 resistant breast cancer cell lines might be different from those in TRA-8 sensitive cell lines as observed in this study, which will be investigated in the future study

In summary, we demonstrated that CaM is recruited into DR5-mediated DISC in a calcium-dependent manner in TRA-8 sensitive MDA-MB-231 or ZR-75-1 breast cancer cells. CaM

antagonist, TFP, inhibited CaM recruitment into DISC, decreased TRA-8 activated DR5 oligomerization, attenuated DISC formation, and reduced TRA-8 activated caspase-dependent apoptotic signaling and TRA-8 induced cytotoxicity in MDA-MB-231 or ZR-75-1 breast cancer cells. These results suggest an important role of CaM in mediating DR5-mediated DISC formation and apoptotic signaling in breast cancer cells. These results suggest that CaM could be a key protein regulator that modulate DR5 mediated apoptosis in breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

CaM	Calmodulin
DR5	Death Receptor 5
Cas-8	Caspase-8
Cas-3	Caspase-3
Cas-9	Caspase-9
PARP	poly ADP ribose polymerase
DISC	Death inducing signaling complex
FADD	Fas associated death domain
TFP	Trifluoperazine
TNF	Tumor necrosis factor
SH2	Src homology domain 2
SHP-1	Tyrosine phosphatase-1

LITERATURE CITED

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FIGURE 1. CaM was recruited into TRA-8 activated DISC in triple-negative MDA-MB-231 and ER-positive ZR-75-1 breast cancer cells

Co-immunoprecipitation of DR5, CaM, FADD and Caspase-8 from MDA-MB-231 (A) and ZR-75-1 cells (B) treated with 1 μg/mL TRA-8 3 hours. **Cell Lysate lane:** whole cell lysate of breast cancer cells before IP. **IP: DISC lane:** Co-IP of CaM with DR5-mediated DISC from cell lysates using anti-DR5 (D-6) antibody with protein A/G Plus-Agarose beads, mouse iso-IgG conjugated sepharose beads, or 4B-sepharose beads. **IP D-6 lane:** IP using anti-DR5 (D-6) antibody with protein A/G Plus-Agarose beads, **IP IsoIgG lane:** IP using a mouse Iso-IgG conjugated sepharose beads. **IP S lane:** IP result using 4B-sepharose beads. Representative results are from two independent experiments.

FIGURE 2. TRA-8 activated DISC in a calcium dependent manner in triple-negative MDA-MB-231 and ER-positive ZR-75-1 breast cancer cells

Co-immunoprecipitation of DR5, CaM, FADD and Caspase-8 from MDA-MB-231 (A) and ZR-75-1 cells (B) with 1 $\mu\text{g}/\text{mL}$ TRA-8 treatment only or 2 mM Ca^{2+} chelator, EGTA pretreatment for 30 minutes followed by 1 $\mu\text{g}/\text{mL}$ TRA-8 treatment for 3 hours. **Cell Lysate lane:** lysate from cells before IP. **IP: DISC lane:** Co-IP of CaM with DR5-mediated DISC from cell lysates using anti-DR5 (D-6) antibody with protein A/G Plus-Agarose beads. Representative results are from two independent experiments.

FIGURE 3. CaM antagonist, TFP, attenuated CaM recruitment into DR5-mediated DISC and inhibited DR5-mediated DISC formation in MDA-MB-231 and ZR-75-1 breast cancer cells
Co-immunoprecipitation of CaM, DR5, FADD and Caspase-8 from MDA-MB-231 (A) and ZR-75-1 cells (B) with 1 μ g/mL TRA-8 treatment only for 3 hours, or preincubated with 10 μ M TFP for 15 or 30 minutes followed by 3 hours 1 μ g/mL TRA-8 treatment. **Cell Lysate lane:** cell lysates before IP. **IP: DISC lane:** Co-IP of CaM with DR5-mediated DISC from cell lysates using anti-DR5 (D-6) antibody with protein A/G Plus-Agarose beads. Representative results are from two independent experiments.

FIGURE 4. CaM antagonist, TFP, inhibited TRA-8 activated DR5 oligomerization in MDA-MB-231 and ZR-75-1 breast cancer cells

(A) Representative fluorescence microscopic images of Cy5.5 labeled TRA-8 on MDA-MB-231 or ZR-75-1 cells with or without 10 μM TFP treatment for 24 hours, when the same color scale was applied for all images. The fluorescence images were overlapped with bright field images showing the cell morphology. (B) Gaussian curves fitting to the distribution of Cy5.5-TRA-8 on the representative cells indicated with white arrows in (A). (C and D) Peak amplitudes of TRA-8/DR5 oligomers when cells were treated with Cy5.5-TRA-8 alone or in combination with TFP in MDA-MB-231 (C) and ZR-75-1 (D) cell lines, respectively.

FIGURE 5. CaM antagonist, TFP or Ca^{2+} chelator, EGTA decreased DR5-mediated caspase cleavage for apoptosis in MDA-MB-231 and ZR-75-1 breast cancer cells
 Western blot analyses of MDA-MB-231 (A) and ZR-75-1 cells (B) for caspase-8, caspase-9, caspase-3, PARP and their cleavage with the cells untreated, treated with 0.5 $\mu\text{g/mL}$ TRA-8 only for 3 hours, treated with 10 μM TFP or 2mM EGTA only for 30 minutes, or treated with 10 μM TFP or 2 mM EGTA for 30 minutes followed by 0.5 $\mu\text{g/mL}$ TRA-8 treatment for 3 hours. Representative results are from two independent experiments.

FIGURE 6. CaM antagonist, TFP, inhibits TRA-8 induced cytotoxicity in MDA-MB-231 and ZR-75-1 breast cancer cells

ATPLite cell viability assay of MDA-MB-231 (A) and ZR-75-1 cells (B) with the cells treated with 0.5 μ g/mL TRA-8 only for 3 hours, or 10 μ M TFP only for 30 minutes or 10 μ M TFP only for 30 minutes followed by 0.5 μ g/mL TRA-8 for 3 hours. For ATPLite cell viability assay, percent of cell viability represents the percent of ATP amount relative the no treatment control. Representative results are from two independent experiments.