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Capacitative calcium entry contributes to the differential transactivation of the epidermal growth factor receptor in response to Thiazolidinediones[§]

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Running title: Role of capacitative calcium entry in EGFR transactivation.

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Abbreviations: PPAR, peroxisome proliferator-activated receptor; MAPK, mitogen-activated protein kinase; TZD, thiazolidinedione; Erk, extracellular signal-regulated kinase; EGFR, epidermal growth factor receptor; SFK, Src family kinases; CSK, c-terminal Src kinase; Pyk2, proline-rich tyrosine kinase; ER, endoplasmic reticulum; BAPTA-AM, 1,2 bis(*o*-aminophenyoxy)ethane-N,N,N'N'-tetraacetic acid; CCE, capacitative calcium entry.

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Abstract

Thiazolidinediones (TZDs) are synthetic ligands for the peroxisome proliferator-activated receptorγ (PPARγ), but also elicit PPARγ-independent effects, most notably activation of MAPKs. Ciglitazone rapidly activates Erk MAPK, an event requiring c-Src kinase-dependent EGFR transactivation while troglitazone, only weakly actives Erk and does not induce EGFR transactivation; the mechanism underlying this difference remains unclear. In this study both ciglitazone and troglitazone increased Src activation. Similar effects were observed with Δ2-derivatives of each TZD, compounds that bind PPARγ but do not lead to its activation, further indicating a PPARγ-independent mechanism. Neither EGFR kinase nor Pyk2 inhibition prevented Src activation; however, inhibition of Src kinase activity prevented Pyk2 activation. Intracellular calcium chelation blocks TZD-induced Pyk2 activation; here, Src activation by both TZDs and ciglitazone-induced EGFR transactivation were prevented by calcium chelation. Accordingly, both TZDs increased calcium concentrations from intracellular stores, however only ciglitazone produced a secondary calcium influx in the presence of extracellular calcium. Removal of extracellular calcium or inhibition of capacitative calcium entry by 2-APB prevented ciglitazone-induced EGFR transactivation and Erk activation, but did not affect upstream kinase signaling pathways. These results demonstrate that upstream kinases (i.e. Src and Pyk2) are required, but not sufficient for EGFR transactivation by TZDs. Moreover, influx of extracellular calcium through capacitative calcium entry may be an unrecognized component that provides a mechanism for the differential induction of EGFR transactivation by these compounds.

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Thiazolidinediones (TZDs) were some of the first drugs developed to address the basic problem of insulin resistance associated with type 2-diabetes. Following their discovery, TZDs were shown to be ligands of the nuclear transcription factor peroxisome proliferator-activated receptor γ (PPARγ) (Lehmann et al., 1995). The insulin-sensitizing effects of TZDs have largely been attributed to their PPARγ-dependent transcription of genes regulating glucose and lipid metabolism in adipose tissue (reviewed in (Yki-Jarvinen, 2004)). In addition to their ability to promote insulin sensitivity, multiple cell and animal studies have shown that TZDs exert growth inhibitory effects (Demetri et al., 1999; Elstner et al., 1998; Kubota et al., 1998; Tsubouchi et al., 2000), actions thought to be independent of PPARγ. Studies have shown that troglitazone inhibited cellular proliferation equally in both PPARγ+/+ and PPARγ-/- mouse embryonic fibroblasts (Palakurthi et al., 2001) and structural derivatives of ciglitazone and troglitazone, that do not induce PPARγ activation, where shown to maintain similar effects on cell growth and signaling pathways as their parent compounds (Shiau et al., 2005). Therefore, TZDs have PPARγ-dependent and independent effects.

Recent work from our lab and others has shown that TZDs mediate the activation of mitogen activated protein kinases (MAPKs), important intracellular signal proteins playing significant roles in coordinating a variety of cellular processes such as cell growth and differentiation, and under some conditions cellular apoptosis (reviewed in(Gardner et al., 2005a)). Specifically, ciglitazone and troglitazone affected two distinct kinase signaling cascades culminating in the activation of either extracellular signal-regulated kinase (Erk) or p38 MAPK. These effects were maintained by structural derivatives lacking the ability to activate PPARγ, further demonstrating a PPARγ-independent mechanism (Gardner et al., 2005b). Interestingly, while both ciglitazone and troglitazone were effective activators of the p38 pathway, only ciglitazone was shown to

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mediate activation of Erk, an event requiring Src kinase mediated epidermal growth factor receptor (EGFR) transactivation (Gardner et al., 2003). The underlying mechanism involved in the differential activation of the EGFR/ Erk MAPK pathway by ciglitazone is not well understood, but could be due to the ability of these compounds to affect upstream signaling, such as Src kinase.

c-Src, the first proto-oncogene discovered, is a member of the Src family kinases (SFKs), a subclass of membrane-associated non-receptor tyrosine kinases involved in a variety of cellular signaling pathways. SFKs are activated in response to cellular signals that promote proliferation, survival, motility, and invasiveness, including activation of cytokine receptors, receptor protein tyrosine kinases, G-protein coupled receptors and integrins (Thomas and Brugge, 1997). Activity of this kinase is negatively regulated by phosphorylation of tyrosine 527 (Tyr^{527}) in the C-terminal tail region of the protein by the closely homologous, C-terminal Src kinase (CSK) (Okada and Nakagawa, 1988). Dephosphorylation of this site results in an intramolecular conformational change and subsequent autophosphorylation within the kinase activation loop, thereby inducing maximal kinase activity. Once active, c-Src phosphorylates multiple substrates involved in a variety of cellular events. Beyond their PPARγ ligand-binding activity, the ability of TZDs to influence cellular signaling pathways may be due to their regulation of Src and/or Src-regulated events.

The EGFR is a single membrane spanning, receptor tyrosine kinase known to affect apoptosis, cell migration and differentiation, adhesion and proliferation (Carpenter et al., 1978; Prenzel et al., 2001). Activation occurs when extracellular ligand (*i.e.*, epidermal growth factor or EGF) binds to the EGFR leading to receptor autophosphorylation on multiple tyrosine residues, followed by activation of downstream kinase signaling cascades (Ullrich and

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Schlessinger, 1990). However, receptor activation can also occur in the absence of physiological ligands via a mechanism termed EGFR 'transactivation'. Proteolytic cleavage of EGF-like ligands by matrix metalloproteinases, nonreceptor tyrosine kinases, stress factors, cell adhesion, G-protein coupled receptors and cytokine receptor have all been associated with EGFR transactivation (Wetzker and Bohmer, 2003). As the EGFR has emerged as a critical transducer of intracellular signals the mechanism(s) regulating transactivation are important to understand.

In the current study we demonstrate that ciglitazone, but not troglitazone induces a large secondary calcium influx from extracellular sources and that removal of this calcium prevents ciglitazone induced EGFR transactivation and Erk activation. Neither Src nor Pyk2, were affected by extracellular calcium removal, but their activation in response to both TZDs was prevented by chelation of ER derived calcium. Collectively these data suggest both ciglitazone and troglitazone modulate the required signals upstream of EGFR transactivation, but that only ciglitazone is capable of inducing EGFR transactivation through its differential modulation of extracellular calcium.

Materials and Methods

Materials

The TZDs ciglitazone, troglitazone (Biomol), Δ2-Ciglitazone and Δ2-troglitazone (described in (Shiau et al., 2005)) were prepared as stock solutions in dimethyl sulfoxide (DMSO). Human recombinant EGF was purchased from Invitrogen. PD153035, PP2, and PP3 were all purchased from Calbiochem. BAPTA-AM was from Molecular Probes. Sodium orthovanadate was purchased from Sigma. Anti-Src monoclonal antibody (B-12) and anti-phosphotyrosine (pan) (PY99) monoclonal antibody were purchased from Santa Cruz Biotechnology. Anti-phospho-EGFR (Tyr^{845}) and (Tyr^{1068}), anti-phospho-Src (Tyr^{416}) and (Tyr^{527}) were purchased from Cell

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Signaling. Anti-EGFR C-terminal polyclonal antibody (#22) and anti-Pyk2 C-terminal polyclonal antibody (#72) were generated as previously described (Li et al., 1998, Yu, 1996 #20). [^{32}P]-γ-ATP was purchased from PerkinElmer Life Sciences.

Cell Culture

The rat liver epithelial cell line GN4, were grown in Richter's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/ streptomycin/ amphotericin B as described previously (Earp et al., 1995). Cells at 70-80% confluence were serum-starved in Richter's minimum essential medium containing 0.1% fetal bovine serum 24 hours prior to experimental treatment.

Cell Lysate Preparation

Following cell stimulation for times indicated, media was removed and cells were quickly washed twice with ice-cold PBS. The cells were then scraped in ice-cold RIPA buffer (150 mM NaCl, 9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS, pH 7.4) with freshly added 100 μM Na_3VO_4, 250 μM phenylmethylsulfonyl fluoride, 5 μg/ ml leupeptin, and 10 nM microcysteine. Cell lysates were centrifuged at 14,000 rpm for 10 minutes at 4°C to removed cellular debris. Protein concentration of the remaining supernatant was determined using the Coomassie protein assay reagent (Pierce).

Immunoblotting

In a typical experiment, 10-30 μg of cell lysate was resuspended in SDS-PAGE sample buffer (0.5 M Tris, pH 6.8, 4.0% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.1% bromphenol blue) and heated for 5 minutes at 95°C in order to denature proteins. The lysates were then resolved by SDS-PAGE on 10% polyacrylamide gels and then transferred to polyvinylidene fluoride (Immobilon-P; Millipore). The immunoblots were incubated in blocking buffer (TBST

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+ 3.0% gelatin) for 1 hour, followed by the appropriate primary antibody overnight at 4°C. Blots were then washed three times with TBST followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1-2 hours at RT. Immunoblots were developed with ECL (Amersham Biosciences) according to the manufacture's instructions and visualized by autoradiography (Kodak X-Omay Blue Film). For some immunoblots, membranes were stripped in buffer (62.5 mM Tris, pH 6.8, 2.0% SDS, 100 mM β-mercaptoethanol) at 55°C for 30 minutes and then reprobed.

Immunoprecipitation

After stimulation the cells were rinsed as described above and scraped into ice-cold RIPA buffer without SDS and then cleared by centrifugation. Various amounts of protein (between 150-500 μg depending on protein) were immunoprecipitated by overnight incubation with 1º antibody at 4ºC with agitation followed by an additional 1-hour incubation after the addition of 20 μL of protein A-agarose bead slurry (Santa Cruz Biotechnology). Immune complexes were collected by brief centrifugation and then washed three times in cold lysis buffer and one time in PBS. Following the last wash, the remaining buffer was removed with a Hamilton syringe, the immune complexes resuspended in SDS-PAGE sample buffer and then separated by SDS-PAGE as described above.

In Vitro Src Kinase Assay

Src kinase activity was measured using a standard commercial Src assay kit (Upstate Biotechnology, Inc.). Briefly, Src was immunoprecipitated as described above from 200 μg of cell lysate by overnight incubation with anti-Src monoclonal antibody (B-12). Src activity in immune complexes was assessed by measuring the transfer of the γ-phosphate of [^{32}P]-γ-ATP to

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a specific Src substrate peptide for 15 minutes at 30°C. Phosphorylated substrate was spotted onto P-81 phosphocellulose paper (Whatman) and quantified with a scintillation counter.

Intracellular calcium measurements

GN4 cells were plated on cover slips overnight in normal growth media as described previously. The media was removed and Fura-2 acetoxymethyl ester (AM) (Molecular Probes Inc., Eugene, OR) was added in HBSS at a final concentration of 2.5 μM and incubated for 30 minutes at room temperature. The cells were washed and incubated for an additional 30 minutes at room temperature in HBSS containing calcium to allow for complete de-esterification. The cover slip was then placed in a gravity-fed perfusion chamber containing HBSS. Changes in intracellular calcium concentration following addition of stimulus were monitored in at least 4 viable cells for each experiment in cells perfused with HBSS with or without calcium. Changes in fluorescence intensity of Fura-2 at excitation wavelengths 340 and 380 nm were monitored using a dual-wavelength fluorescence imaging system (Intracellular Imaging Inc., Cincinnati, OH).

Results

Both ciglitazone and troglitazone increased c-Src phosphorylation and activity independent of PPARγ

Previously we showed that PPAR α and γ agonists increased the phosphorylation of the MAPK Erk in the rat liver epithelial cell line, GN4, an event that required Src-dependent EGFR transactivation (Gardner et al., 2003). Interestingly, ciglitazone but not troglitazone, a related TZD family member, was capable of inducing EGFR transactivation. To investigate the differential effects of these compounds on EGFR transactivation we examined changes in Src tyrosine phosphorylation following exposure of GN4 cells to ciglitazone or troglitazone. The

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ability of Δ2-derivatives of these compounds, which bind but do not activate PPARγ, to activate Src was also examined. Autophosphorylation of Src on tyrosine 416 (Tyr^{416}), which is known to correlate well with elevated levels of Src kinase activity, increased following treatment of cells with ciglitazone, troglitazone or their respective Δ2-derivatives (Figure 1A and B). Src Tyr^{416} phosphorylation was maximal at 5 and 10 minutes following ciglitazone treatment; Δ2-ciglitazone was slower and showed maximal activation around 10 minutes, consistent with previous studies (i.e. EGFR transactivation, Erk phosphorylation) (Gardner et al., 2003). Unexpectedly, both troglitazone and Δ2-troglitazone similarly increased Src Tyr^{416} phosphorylation, with maximal activation occurring at 10 minutes (Figure 1B).

We further examined the phosphorylation status of Src Tyr^{527}, the inhibitory phosphorylation site. Src was highly phosphorylated on Tyr^{527} in serum-starved cells; however following treatment of cells with ciglitazone, troglitazone, or their respective Δ2-derivatives, a time-dependent decrease in Src Tyr^{527} phosphorylation was observed. Ciglitazone noticeably reduced Src Tyr^{527} phosphorylation after 10 minutes and maximally at 30 minutes when compared to vehicle (DMSO) treated cells; again the response obtained with Δ2-ciglitazone was slightly slower when compared to ciglitazone (Figure 1A). Both troglitazone and Δ2-troglitazone decreased Src Tyr^{527} phosphorylation in a comparable time-dependent manner, with maximal loss occurring at 30-minutes (Figure 1B).

Because ciglitazone, troglitazone and their Δ2-derivatives affected phosphorylation events involved in regulation of Src activation, we examined the effects of these compounds on Src kinase activity. Src was immunoprecipitated from cell lysates, and *in vitro* kinase assays performed using a Src-specific substrate peptide. Following a 10-minute treatment, ciglitazone, troglitazone and their Δ2-derivatives significantly increased Src kinase activity approximately 3-

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fold over DMSO vehicle controls (Figure 1C). Treatment of cells for 30 minutes, where Src Tyr^{527} loss was maximal, resulted in a 6-fold increase in Src kinase activity over DMSO vehicle controls for ciglitazone and Δ2-ciglitazone, while troglitazone and Δ2-troglitazone increased Src activity 7-fold over DMSO controls (Figure 1C). Collectively, these data show that ciglitazone, troglitazone and their respective Δ2-derivatives, affect key regulatory phosphorylation sites in Src kinase and increase Src kinase activity. These data further suggest that additional mechanisms are required for EGFR transactivation, as both TZDs increase Src activation, but only ciglitazone is capable of mediating EGFR phosphorylation (Gardner et al., 2005b).

EGFR kinase activity is not involved in Src activation by TZDs

Some studies suggest that EGF stimulated EGFR activation can mediate Src kinase activation; EGF stimulated Jak/ STAT activation required Src kinase activity, while others showed more directly that EGF stimulated EGFR activation mediated an increase in Src activity through the GTPase Ral (Goi et al., 2000; Olayioye et al., 1999). However, our previous data suggested that Src was a required kinase involved in the mechanism of EGFR transactivation in response to TZD treatment. To eliminate the possibility that the EGFR played a role in the mechanism mediating Src activation following TZD treatment, GN4 cells were pretreated with the EGFR kinase inhibitor PD153035 and Src Tyr^{416} phosphorylation in response to TZD treatment was examined. Similar to previous data, both ciglitazone and Δ2-ciglitazone treatment increased EGFR phosphorylation and this was blocked by PD153035 (data not shown) (Gardner et al., 2003). However, PD153035 pretreatment did not affect the ability of ciglitazone or troglitazone to increase Src Tyr^{416} phosphorylation (Figure 2A) demonstrating that EGFR kinase activity was not required for TZD-induced Src activation. Pretreatment of cells with PD153035

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reduced overall EGFR tyrosine phosphorylation stimulated by EGF, demonstrating the effectiveness and EGFR-kinase specificity of this compound in these experiments (Figure 2B).

Inhibition of protein tyrosine phosphatases prevents loss of Tyr^{527} phosphorylation

Src kinase activity is negatively regulated through phosphorylation of the Tyr^{527} residue in the c-terminal region of the protein. To determine if a PTPase was involved in the mechanism of activation of Src by TZDs, we pretreated cells with either 50 μM H_2O_2, sodium orthovanadate or vanadyl hydroperoxide [$V^{(4+)}$-OOH] (pervanadate). Pervanadate, a mixture of sodium orthovanadate and hydrogen peroxide, has been shown to be a more potent inhibitor of PTPase activity than sodium orthovanadate and penetrates cells more readily than sodium orthovanadate or H_2O_2 alone in intact cells (Kadota et al., 1987; Trudel et al., 1991). Following treatment with ciglitazone, troglitazone or their respective Δ2-derivatives, Src Tyr^{527} phosphorylation was decreased (Figure 3A and B). Pretreatment of cells with both H_2O_2 and sodium orthovandate did not affect basal Src Tyr^{527} phosphorylation and there was no inhibitory effect of either pretreatment on TZD-induced Src Tyr^{527} dephosphorylation. However, pervanadate pretreatment completely blocked the dephosphorylation of Src Tyr^{527} following TZD treatment (Figure 3A and B) suggesting that PTPase activity was required for Src Tyr^{527} dephosphorylation and subsequent Src activation.

We further examined the effects of PTPase inhibition by pervanadate on Src kinase activity in cells treated with TZDs or their respective Δ2-derivatives. Following exposure of cells to these compounds, Src kinase activity was increased nearly 6-fold in each condition (Figure 3C), similar to previous results. Pretreatment of cells with either H_2O_2 or sodium orthovanadate did not significantly reduce Src kinase activity; however, TZD-induced Src kinase activity was completely inhibited by pervanadate pretreatment to below basal levels (Figure 3C). These data

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demonstrated that PTPase-mediated Src Tyr^{527} dephosphorylation was required for Src kinase activation following TZD treatment.

Pyk2 phosphorylation is mediated by Src activation

The non-receptor, calcium-dependent proline rich tyrosine kinase, Pyk2, has been implicated in ligand-independent EGFR transactivation (Shah et al., 2003) and has been shown to play a role in the activation of Src. Interestingly, both ciglitazone and troglitazone have been previously shown to increase Pyk2 phosphorylation (Gardner et al., 2003). We therefore examined the role of Pyk2 in mediating both Src activation and EGFR phosphorylation. To prevent Pyk2 activation cells were infected with either an adenoviral C-terminal inhibitory form of Pyk2 (Ad.CRNK) or adenovirus encoding green fluorescent protein (Ad.GFP). As an alternative potential splice variant of Pyk2 (Schaller and Sasaki, 1997), Ad.CRNK expression negatively regulates endogenous Pyk2 autophosphorylation (Li et al., 1999). Addition of 8×10^6 plaque forming units/ mL (the concentration used in these studies) effectively blocked angiotension II-dependent Pyk2 phosphorylation to basal levels (data not shown). As expected, Ad.CRNK overexpression lowered both ciglitazone and troglitazone induced Pyk2 phosphorylation (Figure 4A). In contrast to evidence supporting a role of Pyk2 in mediating Src activation, Ad.CRNK overexpression did not alter increases in Src Tyr^{416} phosphorylation following treatment of cells with ciglitazone or troglitazone (Figure 4B). We further examined EGFR tyrosine phosphorylation. As expected, troglitazone did not affect EGFR Tyr^{845} or Tyr^{1068} phosphorylation; however, ciglitazone increased EGFR phosphorylation on both of these tyrosine residues and this was blocked to near basal levels by Ad.CRNK (Figure 4B). Neither Ad.GFP nor Ad.CRNK affected EGF-stimulated EGFR phosphorylation (data not shown).

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Because inhibition of Pyk2 did not affect Src kinase activation, we examined whether Src was required to mediate Pyk2 activation in response to TZD treatment. When cells were pretreated with the Src kinase inhibitor PP2, ciglitazone and troglitazone-induced increases in Src Tyr^{416} phosphorylation were reduced to basal levels, but were unaffected by PP3 (Figure 5A). Under these conditions, PP2 similarly lowered the increase in Pyk2 phosphorylation following incubation of cells with either ciglitazone or troglitazone (Figure 5B.) Collectively these data show that Pyk2 phosphorylation is mediated by Src activation and additionally, suggest a potential role of Pyk2, in addition to the requirement of Src, in EGFR transactivation.

Both ciglitazone and troglitazone mobilize intracellular calcium

Pyk2 activation is regulated by stimuli that lead to an increase in intracellular calcium concentrations. Moreover other work has demonstrated a rapid increase in Src kinase activity in keratinocytes treated with either a high concentration of extracellular calcium or ionophore (Zhao et al., 1992). Therefore modulation of intracellular calcium could affect the ability of TZDs to activate signaling events. To determine the effects of ciglitazone and troglitazone on changes in intracellular calcium concentrations, GN4 cells were loaded with Fura-2 AM and then challenged with 50 μM ciglitazone or troglitazone. Following administration of ciglitazone or troglitazone in HBSS containing no calcium, we observed a rapid increase in intracellular calcium concentration (Figure 6A and B). These data suggest that the increase in intracellular calcium was due to release from intracellular stores, such as the endoplasmic reticulum.

Effect of intracellular calcium chelation on TZD-induced Src activation

Previous work from our lab showed that other signaling pathways activated in response to ciglitazone or troglitazone were sensitive to intracellular calcium chelation (Gardner et al., 2003). As both compounds were observed to effectively mobilize intracellular calcium, we

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examined if calcium was important to the transactivation of the EGFR and Src kinase activation. Serum-starved cells were pretreated for 20 minutes with 50 μM BAPTA-AM followed by stimulation with DMSO, ciglitazone or troglitazone for 10 minutes times at which maximal Src Tyr^{416} phosphorylation or ciglitazone-induced EGFR transactivation was observed. Ciglitazone and troglitazone increased Src Tyr^{416} phosphorylation after 10 minutes, whereas pretreatment with BAPTA-AM effectively lowered both ciglitazone and troglitazone-induced Src Tyr^{416} phosphorylation (Figure 7A and B). This is consistent with previous data demonstrating that intracellular calcium chelation by BAPTA-AM disrupted Pyk2 activation by ciglitazone and troglitazone (Gardner et al., 2003) and fits with data presented here showing that Src is important to Pyk2 activation. Because BAPTA-AM lowered the activation of these kinases, we further examined the effect of calcium chelation on EGFR phosphorylation. BAPTA-AM pretreatment lowered EGFR Tyr^{845} and Tyr^{1068} phosphorylation following treatment of cells for 10 minutes with ciglitazone, but was without effect on EGF-stimulated EGFR phosphorylation on either of these sites (Figure 7C). Consistent with previous observations, troglitazone did not increase EGFR phosphorylation. These data demonstrate that Src activation and ciglitazone-induced EGFR transactivation are calcium sensitive events, whereas EGF-dependent EGFR autophosphorylation is calcium independent.

Ciglitazone but not troglitazone induces a second increase in intracellular calcium

Depletion of intracellular calcium stores leads to the retrograde process of store-operated calcium entry or capacitative calcium entry (CCE) (Putney, 1986). Our data show that both ciglitazone and troglitazone mediated calcium release in the absence of any extracellular calcium (Figure 6A and B), suggesting that an intracellular store is affected, presumably the ER. We therefore tested the ability of ciglitazone and troglitazone to induce CCE by challenging Fura-2

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loaded GN4 cells with each TZD in the presence of extracellular calcium. Following addition of either ciglitazone or troglitazone there was a rapid increase in intracellular calcium concentration that peaked near 100 nM (Figure 8). This was consistent with our data showing that each TZD lead to an increase in intracellular calcium that peaked around 150 nM in the absence of extracellular calcium, further suggesting that this first peak resulted from depletion of an intracellular store. In the presence of extracellular calcium ciglitazone produced a secondary calcium influx that peaked near 700 nM (Figure 8, black line). By contrast, troglitazone failed to produce this secondary response (Figure 8, red line). These data demonstrate that ciglitazone, but not troglitazone mediated a large secondary increase in intracellular calcium concentration, indicative of CCE.

Removal of extracellular calcium prevents EGFR transactivation and Erk activation

Intracellular calcium chelation with BAPTA-AM lowered both ciglitazone and troglitazone-induced Src activation and reduced ciglitazone-stimulated EGFR transactivation, but only ciglitazone mediated a second sustained increase in calcium. To determine if this secondary influx of calcium was involved in the ability of ciglitazone to induce EGFR transactivation and subsequent Erk activation, cells were stimulated with ciglitazone in the presence and absence of extracellular calcium and EGFR and Erk phosphorylation were examined. In the absence of extracellular calcium the ability of ciglitazone and Δ2-ciglitazone to cause EGFR phosphorylation was blocked, whereas the effect of EGF stimulated EGFR phosphorylation was not affected (Figure 9A). Further, activation of Erk was similarly prevented when cells were stimulated with either ciglitazone or Δ2-ciglitazone in the absence of extracellular calcium. In contrast, the ability of either ciglitazone or troglitazone to induced Src Tyr^{416} phosphorylation was not affected by removal of extracellular calcium (Figure 9B) and moreover, induction of p38

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phosphorylation, an event previously shown to be dependent upon ER-stress derived calcium-dependent CAMK II activation, was also not affected (Figure 9C). Collectively, these data show that extracellular calcium influx is needed for ciglitazone-induced transactivation of the EGFR and subsequent Erk activation, but is not a required element mediating Src kinase or p38 activation, events thought to be linked to ER calcium release.

Inhibition of capacitative calcium entry prevent ciglitazone-induced EGFR transactivation

Because extracellular calcium was observed to be a required for EGFR transactivation following ciglitazone treatment, the possible role of capacitative calcium entry in this process was further investigated. Cells were pretreated with 2-aminoethyldiphenyl borate (2-APB), a known inhibitor of CCE (Braun et al., 2003), followed by ciglitazone, troglitazone or EGF treatment. As expected, ciglitazone but not troglitazone induced EGFR Tyr^{845} and Tyr^{1068} phosphorylation. 2-APB effectively prevented ciglitazone-induced EGFR transactivation, but had little effect on EGFR phosphorylation following EGF treatment (Figure 10A). In contrast, 2-APB did not inhibit ciglitazone or troglitazone-induced Src Tyr^{416} phosphorylation (Figure 10B). These data suggest a role for CCE in EGFR transactivation and subsequent MAPK activation in response to ciglitazone and provide a potential mechanism for the differential effects on MAPK activation observed with these compounds. Moreover, these data demonstrate that extracellular calcium is not required for TZD-induced Src activation.

Discussion

The ability of TZDs, PPARγ ligands, to elicit cellular effects such as cell differentiation and growth inhibition or induction of apoptosis independent of PPAR activation is well documented. Work from our lab and others have demonstrated that TZDs activate members of the MAPKs in different cell models (Gardner et al., 2005a; Lennon et al., 2002; Mounho and Thrall, 1999;

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Rokos and Ledwith, 1997). As MAPK signaling affects an array of transcription factors, leading to gene expression, our lab has sought to define the molecular mechanisms involved in MAPK activation by TZDs. Recent work demonstrated that ciglitazone, but not troglitazone rapidly activated Erk, an effect dependent upon Src-mediated EGFR transactivation (Gardner et al., 2003). In this study we found that ciglitazone and troglitazone effectively increased Src kinase activity independent of PPARγ activation. Thus, we concluded that an undefined mechanism specifically activated by ciglitazone, in addition to Src kinase, was necessary for EGFR transactivation and subsequent Erk activation. We demonstrated that calcium influx from extracellular sources through CCE is a critical factor involved in the differential transactivation of the EGFR by these compounds. Although both TZDs effectively mediated release of calcium from intracellular stores, only ciglitazone induced a large secondary calcium influx. Removal of extracellular calcium or pharmacological inhibition of CCE prevented ciglitazone-induced EGFR transactivation and Erk activation.

Src has been shown to be involved in a diverse array of signaling pathways. Studies have demonstrated a role of Src kinase in EGF stimulated EGFR activation, whereby EGFR–dependent autophosphorylation recruits Src to the receptor thereby allowing Src-dependent Tyr^{845} phosphorylation (Biscardi et al., 1999). Others demonstrated that this event led to enhanced mitogenic response to EGF (Luttrell et al., 1988). Alternately, Src can mediate phosphorylation of the EGFR in the absence of EGF ligand in response to various stimuli. Src-mediated phosphorylation at the Tyr^{845} site is thought induce receptor dimerization leading to EGFR kinase activation and autophosphorylation that is indistinguishable from EGF-stimulated EGFR activation (Prenzel et al., 2001). Previously we demonstrated a required role for Src kinase in EGFR transactivation by PPARα or PPARγ agonists and ciglitazone treatment was

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shown to increase phosphorylation of the EGFR at Tyr^{845} (Gardner et al., 2003). In contrast, troglitazone failed to mediate EGFR phosphorylation and transactivation, a response that could be explained by differential activation of Src kinase by these compounds. Here we demonstrated that both TZDs and Δ2-derivatives of each compound increased Src kinase activity (Figure 1C). Src kinase activity is negatively regulated by phosphorylation of Tyr^{527} in the C-terminal region of the protein by CSK due to an intramolecular interaction of Src's SH2 domain with the phosphorylated Tyr^{527} site (Okada and Nakagawa, 1988). EGF stimulated EGFR activation has been shown to recruit Src into receptor complexes leading to increased Src kinase activity (Olayioye et al., 1999). Further, work in human epidermoid carcinoma cells demonstrated that the Shc adaptor protein was a novel mediator of EGF stimulated Src activation through the EGFR; activation was associated with Src autophosphorylation, but dephosphorylation in the c-terminal tail region was not observed (Sato et al., 2002). Our work demonstrated that both TZDs induced PTPase-mediated dephosphorylation of Tyr^{527} (Figure 1A and B). Inhibition of other signals known to mediate Src activation, such as EGFR or Pyk2 did not prevent TZD-induced Src activation (Figure 2 and 4); however inhibition of PTPase activity completely blocked TZD-induced Src Tyr^{527} dephosphorylation and increases in Src activity (Figure 3). These data show that TZD mediated Src activation involves a PTPase, however the exact mechanisms involved are still unknown. TZDs could mediate changes in the association of Src-specific PTPase or alternatively, affect localization and activity of CSK. Furthermore, these data suggest that in addition to Src, another mechanism must be involved to explain the differential transactivation of the EGFR following TZD treatment.

Pyk2 (also CAKβ/ RAFTK/ CadTK) is a member of the focal adhesion kinase (FAK) family (Avraham et al., 2000), highly expressed in GN4 cells and can be activated by stimuli that

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increase intracellular calcium (Graves et al., 1997) or in response to stress signals (Yu et al., 1996). Both ciglitazone and troglitazone have been shown to increase Pyk2 tyrosine phosphorylation (Gardner et al., 2003). Here, inhibition of Src kinase with PP2 prevented TZD-induced Pyk2 phosphorylation (Figure 5B), but inhibition of Pyk2 phosphorylation with the dominant negative Ad.CRNK did not effect Src Tyr^{416} phosphorylation (Figure 4) suggesting that Src mediated the activation of Pyk2. These data contrast to other work demonstrating that Pyk2 mediates Src activation; specifically Pyk2 autophosphorylation on tyrosine 402 (Tyr^{402}), in response to lysophosphatidic acid (LPA) or bradykinin led to recruitment and binding of Src's SH2 domain, increased Src kinase activity, and subsequent Src-dependent phosphorylation of additional tyrosine residues within Pyk2 (Dikic et al., 1996). However, other work has shown that co-expression of Src with kinase-deficient Pyk2 led to increased Pyk2 tyrosine phosphorylation, demonstrating that Src could phosphorylate Pyk2 directly (Li et al., 1999). Therefore, TZD-induced Src kinase activation may target direct Pyk2 tyrosine phosphorylation and subsequent activation.

In this study, ciglitazone and troglitazone increased intracellular calcium concentrations in the absence of extracellular calcium, suggesting that this initial rise in calcium was due to depletion of intracellular stores (Figure 6). This supports work showing that both ciglitazone and troglitazone transiently increased intracellular calcium in embryonic stem cells; additionally, thapsigargin, a specific inhibitor of the SER-Ca^{2+} ATPase, known to cause ER calcium store depletion, did not further induce Ca^{2+} release indicating that TZDs were affecting ER calcium stores (Palakurthi et al., 2001). Previous work from our laboratory also suggests that TZDs affect ER calcium in that ciglitazone and troglitazone rapidly activated classical markers of ER stress including PERK (protein kinase R-like endoplasmic reticulum kinase), PKR (double-

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stranded RNA-activated protein kinase) and eIF-2α (eukaryotic translation initiation factor 2α) (Gardner et al., 2005b), events known to correlate with ER calcium depletion (Kuang et al., 2005).

A variety of intracellular signaling pathways are regulated by changes in intracellular calcium (Berridge et al., 2000). Previously we demonstrated that Pyk2 activation following TZD treatment was sensitive to calcium chelation (Gardner et al., 2003). Similarly, calcium chelation with BAPTA-AM reduced TZD-induced Src Tyr^{416} phosphorylation (Figure 7A). With data demonstrating that Src acts as an upstream kinase mediating Pyk2 activation in response to TZD treatment, these additional data suggest that Src may be the initial calcium-activated event mediating Pyk2 activation. The exact mechanism whereby calcium mediates Src activation is unclear, but others have shown that elevation of intracellular calcium mediated increased Src kinase activity (Zhao et al., 1992). Our data suggests that an internal cellular source of calcium is important to the mechanism of Src activation as removal of extracellular calcium did not effect TZD-stimulated Src kinase activation (Figure 9B), but intracellular calcium chelation did. This agrees with other work in which thrombin, a growth factor known to mobilize intracellular calcium stores through generation of inositol trisphosphate (IP_3), or thapsigargin led to an increase in Src Tyr^{416} phosphorylation (Bobe et al., 2003). Thus, these data clarify that intracellular calcium store release could play a role as the initial signaling event leading to Src kinase and subsequent Pyk2 activation.

CCE (also described as store-operated calcium entry) is a regulated mechanism of calcium entry in non-excitable cells (Putney, 1986). Depletion of intracellular calcium stores through the actions of IP_3, SERCA inhibition by thapsigargin, or other ER calcium releasing signals, activates a pathway leading to the opening of plasma membrane calcium channels allowing for

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influx of calcium from extracellular sources. When GN4 cells were exposed to ciglitazone in the presence of extracellular calcium, we observed a large secondary influx of calcium following an initial rise in intracellular calcium. While troglitazone similarly produced the initial rise in calcium, there was no secondary response observed (Figure 8). In agreement with our data, ciglitazone has been shown to inhibit cell proliferation in leiomyoma cells through activation of CCE (Kim et al., 2005), while other work has demonstrated that troglitazone actually prevented CCE in aortic endothelial cells (Kawasaki et al., 1999). Therefore, the differential effects of these compounds on CCE could contribute to the difference observed in EGFR transactivation.

Other works have highlighted the importance of calcium in the mechanism of EGFR transactivation. Our data support these observations showing that BAPTA-AM reduced EGFR transactivation following ciglitazone treatment (Figure 7C). Additionally, activation of kinases thought to be required for EGFR transactivation was also reduced by calcium chelation. Troglitazone activated upstream kinase signals necessary for EGFR transactivation and depleted ER calcium stores, but failed to transactivate the EGFR. Interestingly, our data demonstrated that troglitazone failed to mediate CCE, suggesting that the secondary influx of calcium by CCE was important to the mechanism of EGFR transactivation. When cells were treated with ciglitazone, which produced a secondary calcium influx (Figure 8), in the absence of any extracellular calcium, EGFR transactivation was prevented (Figure 9A). This agrees with previous studies which demonstrated that treatment of cells with a calcium ionophore could induce EGFR phosphorylation (Eguchi et al., 1998; Rosen and Greenberg, 1996). Further, bradykinin-induced EGFR transactivation was shown to be prevented by extracellular calcium chelation using EGTA (Zwick et al., 1997). However, we further hypothesized that in addition to upstream kinase signaling events, the regulated entry of calcium from extracellular sources

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through CCE is an additional requirement in the mechanism of EGFR transactivation. When CCE was prevented by 2-APB, ciglitazone failed to induce EGFR transactivation even though upstream kinases (i.e. Src) were still activated; EGF stimulated EGFR phosphorylation was not affected by 2-APB (Figure 10). Therefore, these data support a required role of CCE in EGFR transactivation and provide an explanation for the difference observed following treatment of cells with TZDs. Since many other stimuli have been shown to induce EGFR transactivation, the requirement of CCE should be further investigated.

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Footnotes

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Figure Legends

Figure 1. Ciglitazone and troglitazone, and their respective Δ2-derivatives alter Src kinase phosphorylation and increase kinase activity. Rat liver epithelial cells (GN4) were grown to confluence and serum-deprived overnight in media containing 0.1% fetal bovine serum. Cells were stimulated with (**A**) 50 μM ciglitazone (Cig), Δ2-ciglitazone (Δ2Cig), or 0.1% DMSO (vehicle control), and (**B**) 50 μM troglitazone (Tro) or Δ2-troglitazone (Δ2Tro) for the times indicated in minutes (min). Cells lysates were prepared and subjected to 10% SDS-PAGE. Src tyrosine phosphorylation was determined by immunoblotting (IB) using either an anti-phospho-Src Tyr^{416} or Tyr^{527} antibodies. The blots were stripped and reprobed using antibodies directed against total Src to determine equal protein loading. **C**, Src kinase activity was measured as described in MATERIALS AND METHODS. Results are mean ± SEM (n=3). * $p< 0.05$, ** $p< 0.001$ for comparison with respective DMSO vehicle control by ANOVA with post hoc comparisons using Tukey's multiple comparisons test.

Figure 2. EGFR kinase activity is not required for Src phosphorylation by TZDs. Confluent GN4 cells were pre-treated with 10 μM PD153035 for 30 minutes. **A**, Cells were treated with 50 μM ciglitazone (Cig), Δ2-ciglitazone (Δ2Cig), troglitazone (Tro) or Δ2-troglitazone (Δ2Tro) or 0.1% DMSO (vehicle control) for 10 minutes. Cell lysates were prepared and subjected to SDS-PAGE. Src tyrosine phosphorylation was determined by immunoblotting (IB) using anti-phospho-Src Tyr^{416} antibody. **B**, cells were treated with or without 100 ng/ mL EGF for 5 minutes. Following immunoprecipitation of EGFR, tyrosine phosphorylation was determined by immunoblotting (IB) using anti-PY99 (phospho-tyrosine) antibody. The blots

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were stripped and reprobed using antibodies directed against total Src or EGFR, respectively to determine equal protein loading.

Figure 3. Inhibition of protein tyrosine phosphatases prevents loss of Src (Tyr^{527}) phosphorylation and blocks Src kinase activity. Cells were grown to confluence, serum-deprived overnight and then pretreated for 5 minutes with either 50 μM H_2O_2, sodium orthovanadate (Na_3VO_4) or pervanadate followed by 30 minutes with (**A**) 50 μM ciglitazone (Cig), Δ2-ciglitazone (Δ2Cig), (**B**) troglitazone (Tro), Δ2-troglitazone (Δ2Tro) or 0.1% DMSO (vehicle control). Cell lysates were prepared and subjected to 10% SDS-PAGE. Src tyrosine phosphorylation was determined by immunoblotting (IB) using anti-phospho-Src Tyr^{527} antibody. The blots were stripped and reprobed using antibodies directed against total Src. **C**, Src kinase activity from treated or untreated samples was determined as previously described. Results are mean ± SEM (n=3). * $p< 0.05$, ** $p< 0.001$ for comparison with respective DMSO vehicle control by ANOVA with post hoc comparisons using Tukey's multiple comparisons test.

Figure 4. Inhibition of Pyk2 prevents ciglitazone induced EGFR transactivation, but not TZD stimulated Src kinase phosphorylation. Following the addition of 8 x 10^6 plaque-forming units/ mL Ad.GFP or Ad.CRNK, near confluent GN4 cells were serum-deprived for 18 hours in 0.1% FBS containing media. Cells were then treated with 50 μM ciglitazone, troglitazone, 50 ng/ mL EGF, or 0.1% DMSO for 10 minutes and lysates prepared. **A**, Pyk2 was immunoprecipitated (IP) and immune complexes were subjected to SDS-PAGE. The effect of Ad.CRNK on Pyk2 phosphorylation was determined by immunoblotting (IB) with a pan anti-phosphotyrosine (PY99) antibody; total Pyk2 was determined using an anti-Pyk2 antibody. **B**,

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total cell lysates were immunoblotted to detect changes in Src Tyr^{416} and EGFR Tyr^{845} and Tyr^{1068} phosphorylation. Equal loading was examined on stripped blots using total Src or EGFR antibodies, respectively.

Figure 5. Src kinase inhibition prevents Pyk2 phosphorylation. GN4 cells grown to confluence were pretreated with 10 μM PP2 or PP3 for 30 minutes followed by 50 μM ciglitazone (Cig) or troglitazone (Tro) or 0.1% DMSO for 10 minutes. Cell lysates were prepared as previously described. **A**, cell lysates were subjected to SDS-PAGE and immunoblotted (IB) with Src Tyr^{416} antibodies. Blots were stripped and reprobed for total Src. **B**, Pyk2 was immunoprecipitated (IP) from lysates and immune complexes were subjected to SDS-PAGE. Phosphorylation of Pyk2 was determined by immunoblot (IB) with a pan anti-phosphotyrosine (PY99) antibody; total Pyk2 was determined using an anti-Pyk2 antibody.

Figure 6. Ciglitazone and troglitazone increase intracellular calcium concentrations by depletion of intracellular stores. GN4 cells were loaded with Fura-2 AM and changes in intracellular calcium concentrations were monitored as described in Experimental Procedures following stimulation of cells with 50 μM ciglitazone or troglitazone in HBSS lacking any calcium. Shown is a representative experiment from 3 separate trials.

Figure 7. Intracellular calcium chelation blunts Src Tyr^{416} phosphorylation and ciglitazone induced EGFR phosphorylation. Serum-starved GN4 cells were pretreated 20 minutes with 50 μM BAPTA-AM. Cells were treated with 50 μM ciglitazone or troglitazone for 10 minutes (min) and EGF (100 ng/ mL) for 5 minutes. **A** and **C**, cell lysates were subjected to SDS-PAGE

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and immunoblotted (IB) with Src Tyr^{416} antibody or anti-phospho-EGFR Tyr^{845} and Tyr^{1068} antibodies. Blots were stripped and reprobed for total protein to determine equal loading. **B**, bands from IB gels were quantified from 3 separate experiments and expressed as Fold Change compared to unstimulated controls (Basal).

Figure 8. The effect of ciglitazone and troglitazone on changes in intracellular calcium concentrations in the presence of extracellular calcium. Fura-2 AM loaded GN4 cells were stimulated with 50 μM ciglitazone or troglitazone in HBSS containing 1 mM calcium as indicated by the arrow and changes in intracellular calcium concentrations were measured as described in Experimental Procedures. Shown is a representative experiment from 3 separate trials.

Figure 9. Removal of extracellular calcium prevents EGFR transactivation and Erk activation, but does not affect upstream signals. Cells were grown to near confluence and then serum-deprived overnight. The media was removed and cells were washed with HBSS and the incubated for 30 minutes in HBSS containing (+) or lacking (-) 1 mM Ca^{2+}. Cells were then exposed to 50 μM ciglitazone (Cig), Δ2-ciglitazone (Δ2Cig), troglitazone (Tro) or Δ2-troglitazone (D2Tro) or 0.1% DMSO (vehicle control) for 10 minutes. Some cells were also treated with 100 ng/ mL EGF for 5 minutes. Cell lysates were prepared and subjected to 10% SDS-PAGE. **A**, EGFR and Erk phosphorylation were determined by immunoblotting (IB) using anti-phospho-EGFR Tyr^{845} and Tyr^{1068} or anti-phospho-Erk antibodies. Blots were stripped and reprobed using antibodies against total EGFR and Erk. **B**, Src tyrosine phosphorylation was determined by IB using anti-phospho-Src Tyr^{416} antibody. **C**, p-38 phosphorylation was

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determined using an anti-phospho-p38 (p-p38) antibody. The blots were stripped and reprobed using total antibodies to its respective protein to determine equal protein loading.

Figure 10. 2-APB blocks EGFR transactivation by ciglitazone. GN4 cells were grown to confluence and serum-starved overnight. Some cells were pretreated with 30 μM 2-APB for 15 minutes followed by 50 μM ciglitazone (Cig) or 50 ng/ mL EGF for 10 minutes. **A**, cell lysates were prepared and EGFR was immunoprecipatated from lysates, immune complexes were subjected to SDS-PAGE, and then immunoblotted (IB) with a pan anti-phosphotyrosine (PY99) antibody. **B**, Src and Erk phosphorylation were determined by immunoblotting (IB) using anti-phospho-Src Tyr^{416} or anti-phospho-Erk antibodies, respectively. Blots were stripped and reprobed for total protein.

Figure 11. Schematic representation of signaling mechanisms involved in differential EGFR transactivation by TZDs. Both ciglitazone and troglitazone mediated a transient increase in intracellular calcium concentration, presumably through ER store release, and increased Src-dependent Pyk2 activation. Intracellular calcium chelation lowered both TZD-induced Src activation and ciglitazone stimulated EGFR transactivation. The mechanism involved in calcium mediated Src activation is not known. Only ciglitazone induced a second influx of calcium; removal of extracellular calcium or inhibition of CCE with 2-APB prevented ciglitazone-induced EGFR transactivation. Moreover, inhibition of Pyk2 also prevented ciglitazone induced EGFR transactivation. While Src has been shown to directly mediate phosphorylation of the EGFR, the mechanisms by which CCE-derived calcium and Pyk2 are involved in mediating EGFR transactivation remain unclear.

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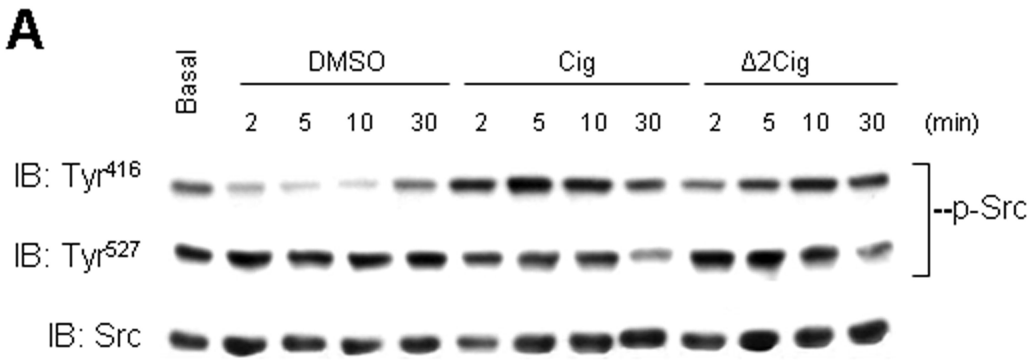
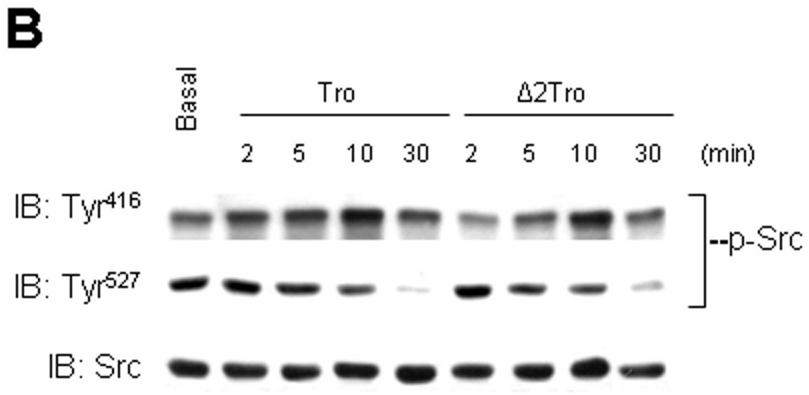
FIGURE 1





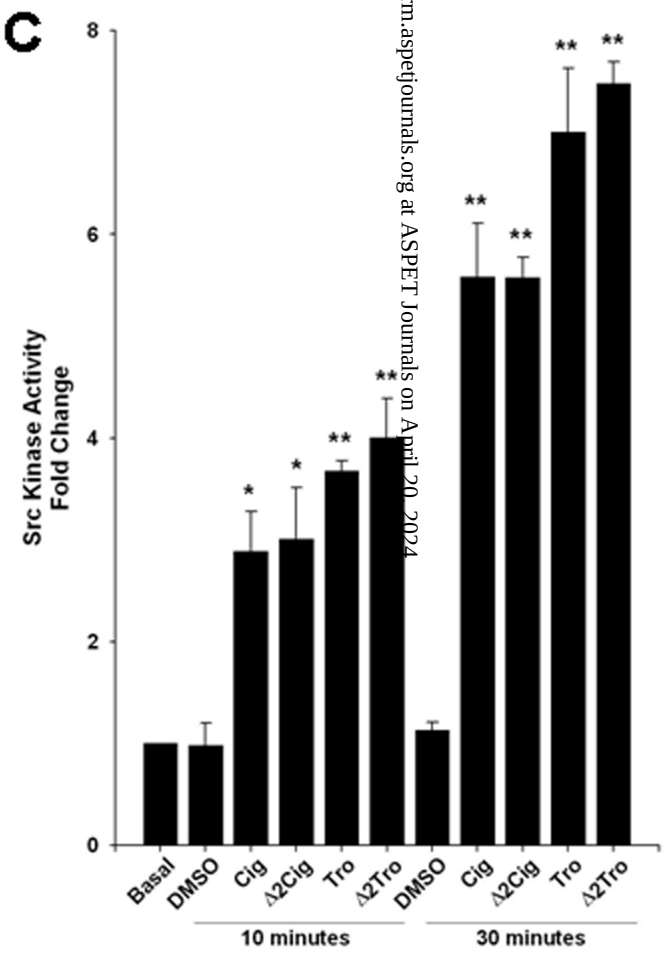


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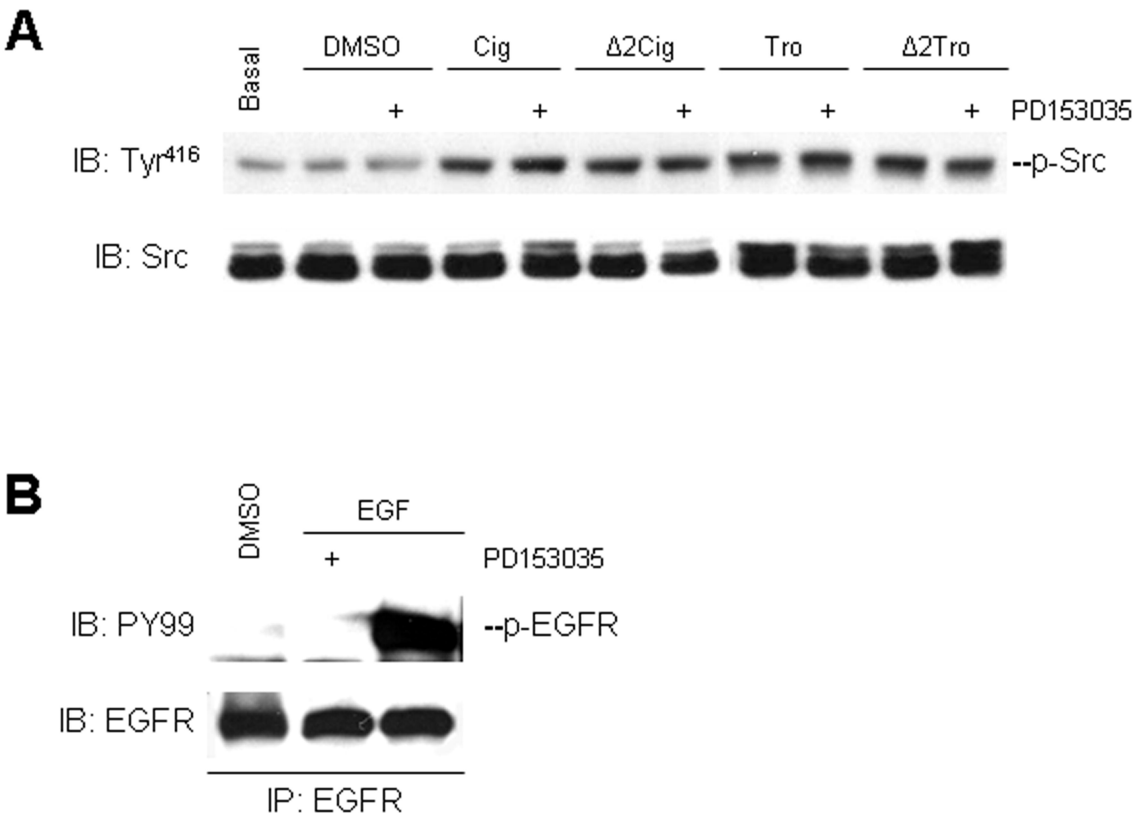
FIGURE 2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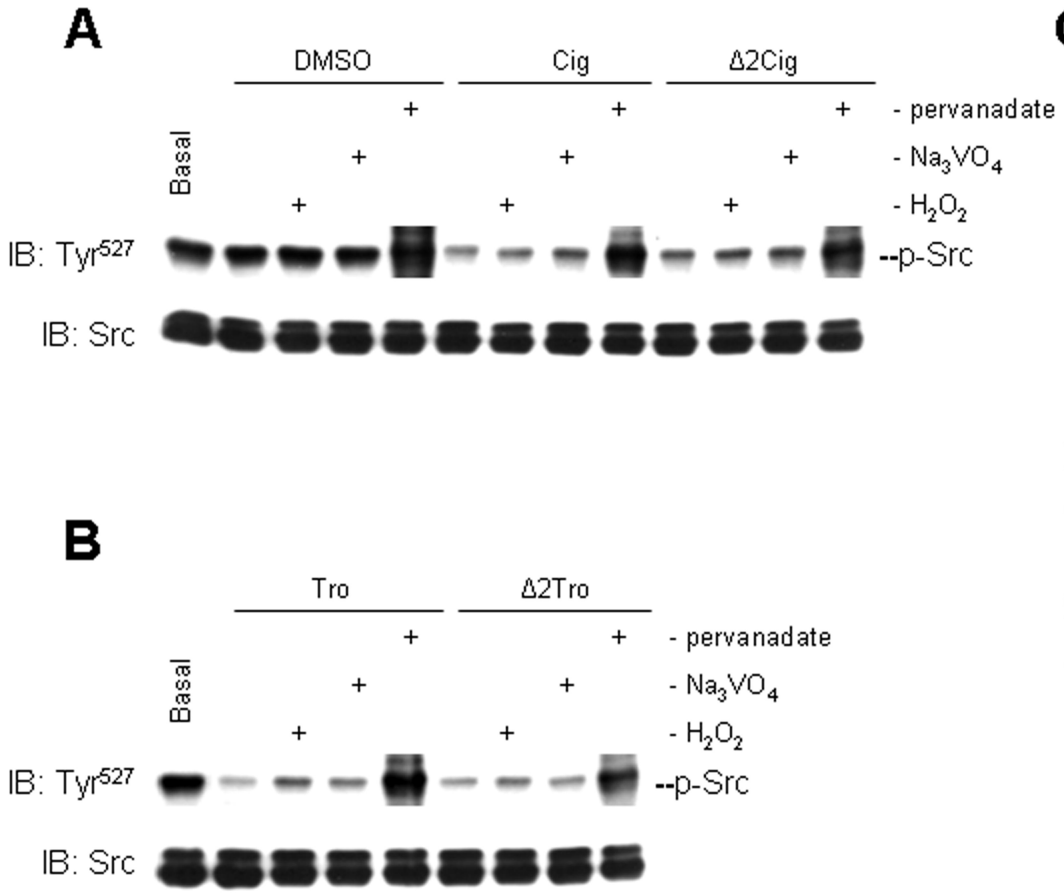
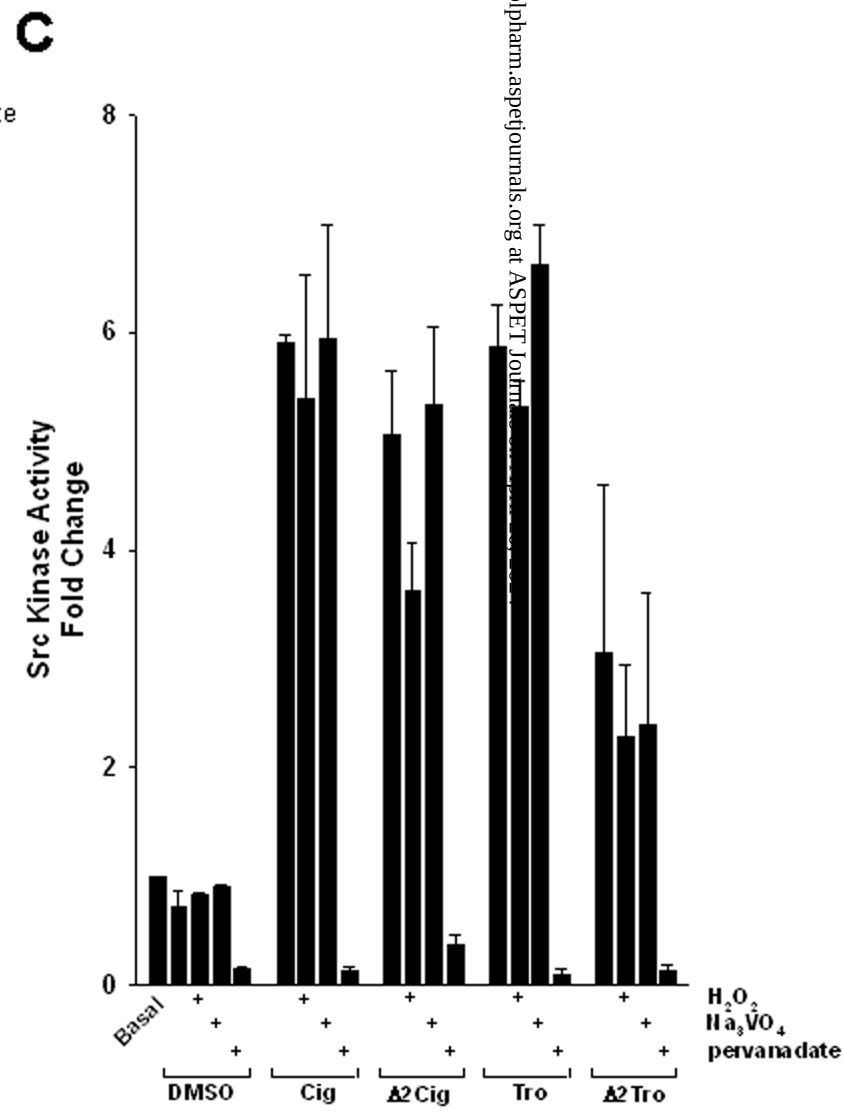
Basal
DMSO
Cig
Δ2Cig
Tro
Δ2Tro
+
+
+
+
+
PD153035
IB: Tyr416
--p-Src
IB: Src
B
DMSO
EGF
+
PD153035
IB: PY99
--p-EGFR
IB: EGFR
IP: EGFR

FIGURE 3





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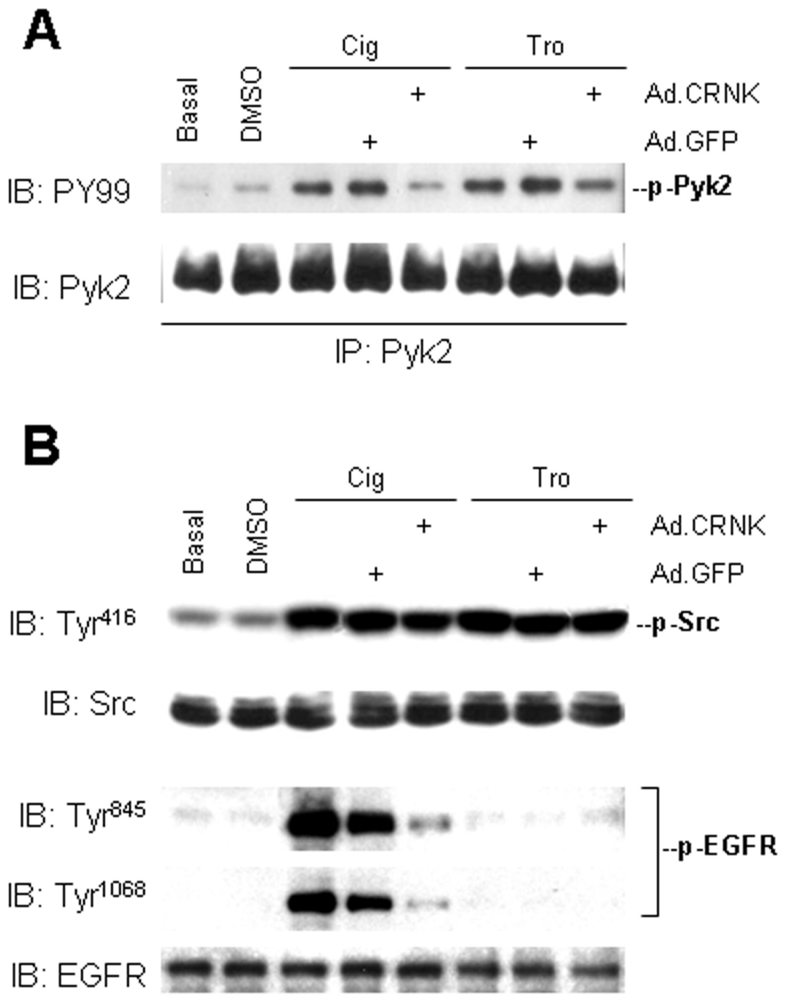
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Basal
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Cig
Tro
+
+
+
+
Ad.CRNK
Ad.GFP
IB: PY99
--p-Pyk2
IB: Pyk2
IP: Pyk2
B
Basal
DMSO
Cig
Tro
+
+
+
+
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IB: Src
IB: Tyr845
IB: Tyr1068
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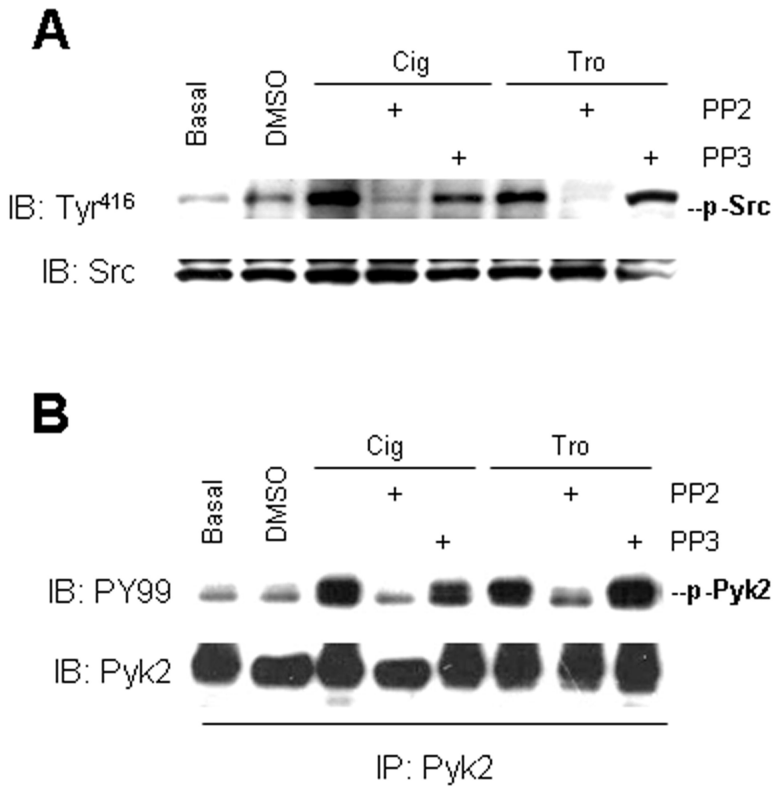
IB: EGFR

FIGURE 5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Cig
Tro
+
+
PP2
+
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PP3
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DMSO
Cig
Tro
+
+
PP2
+
+
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IB: Pyk2
IP: Pyk2

FIGURE 6

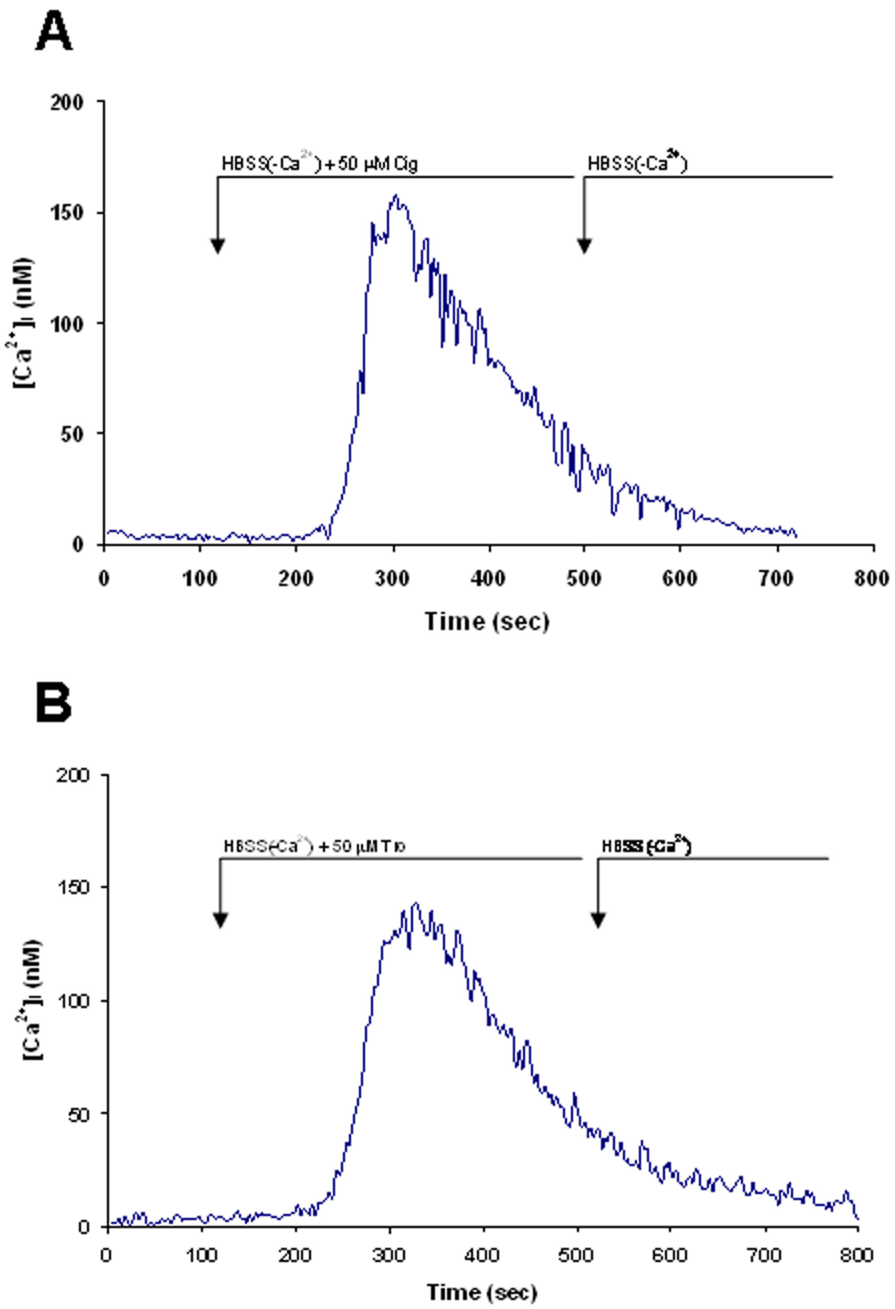

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FIGURE 7

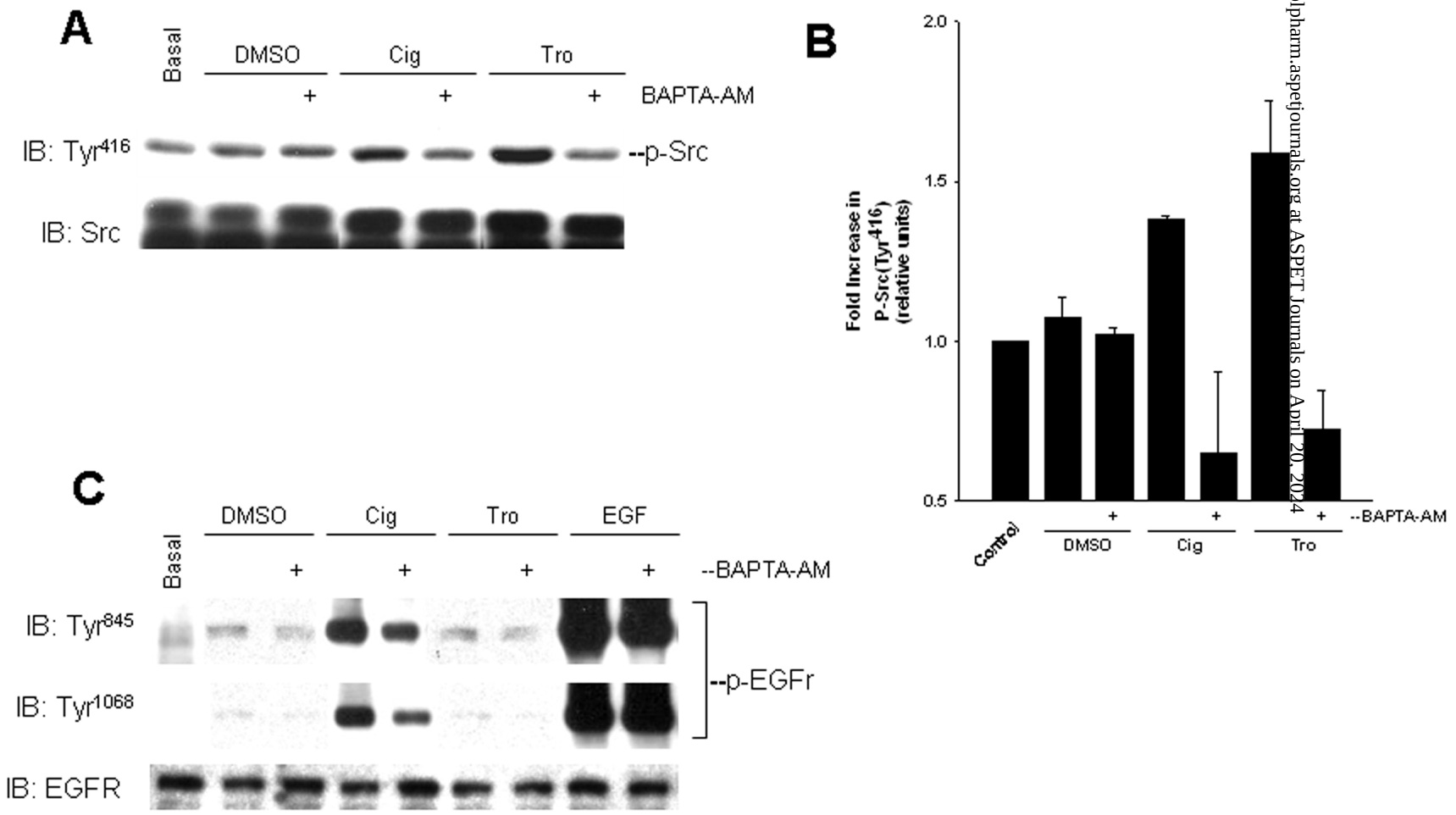

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FIGURE 8

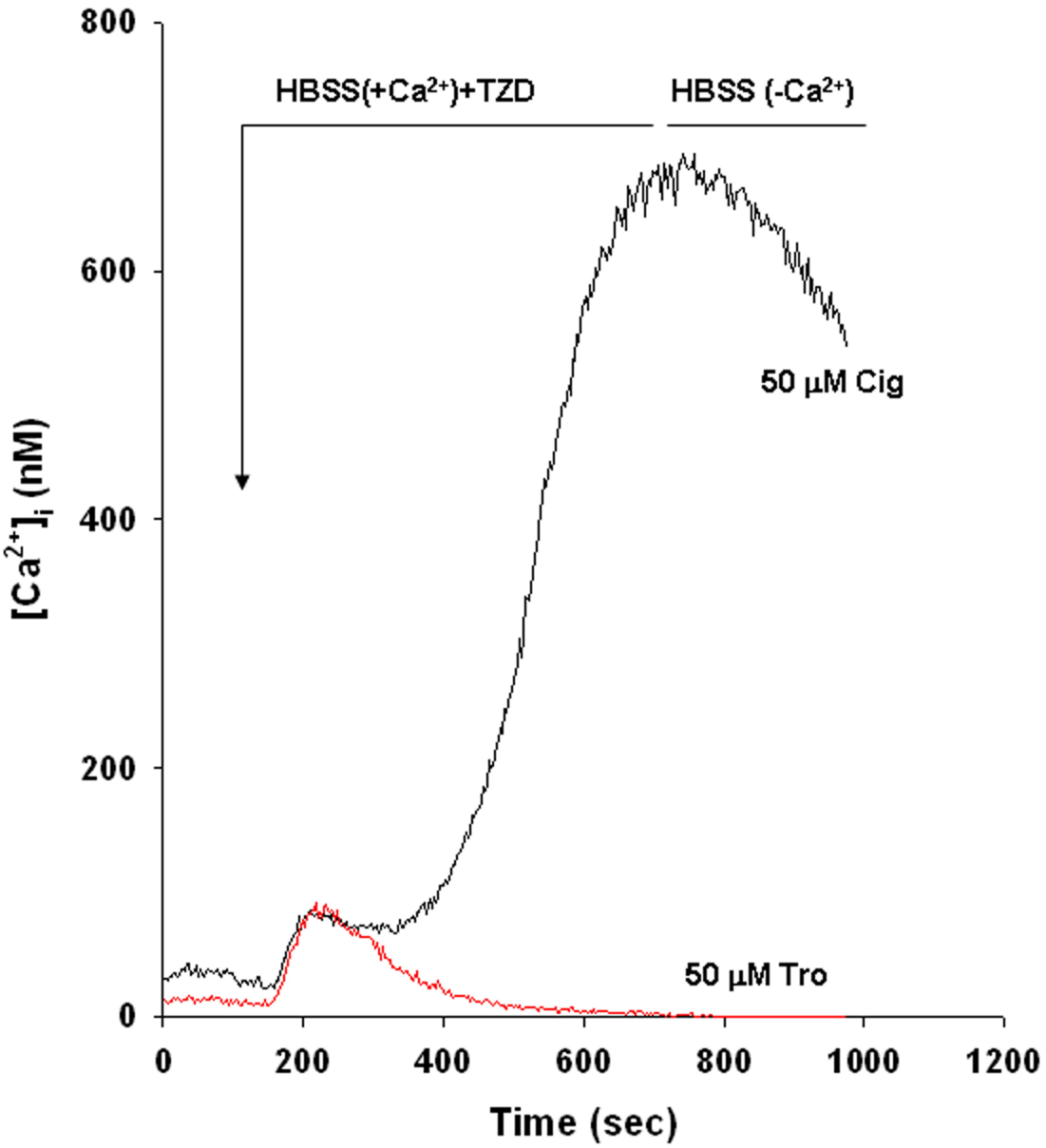
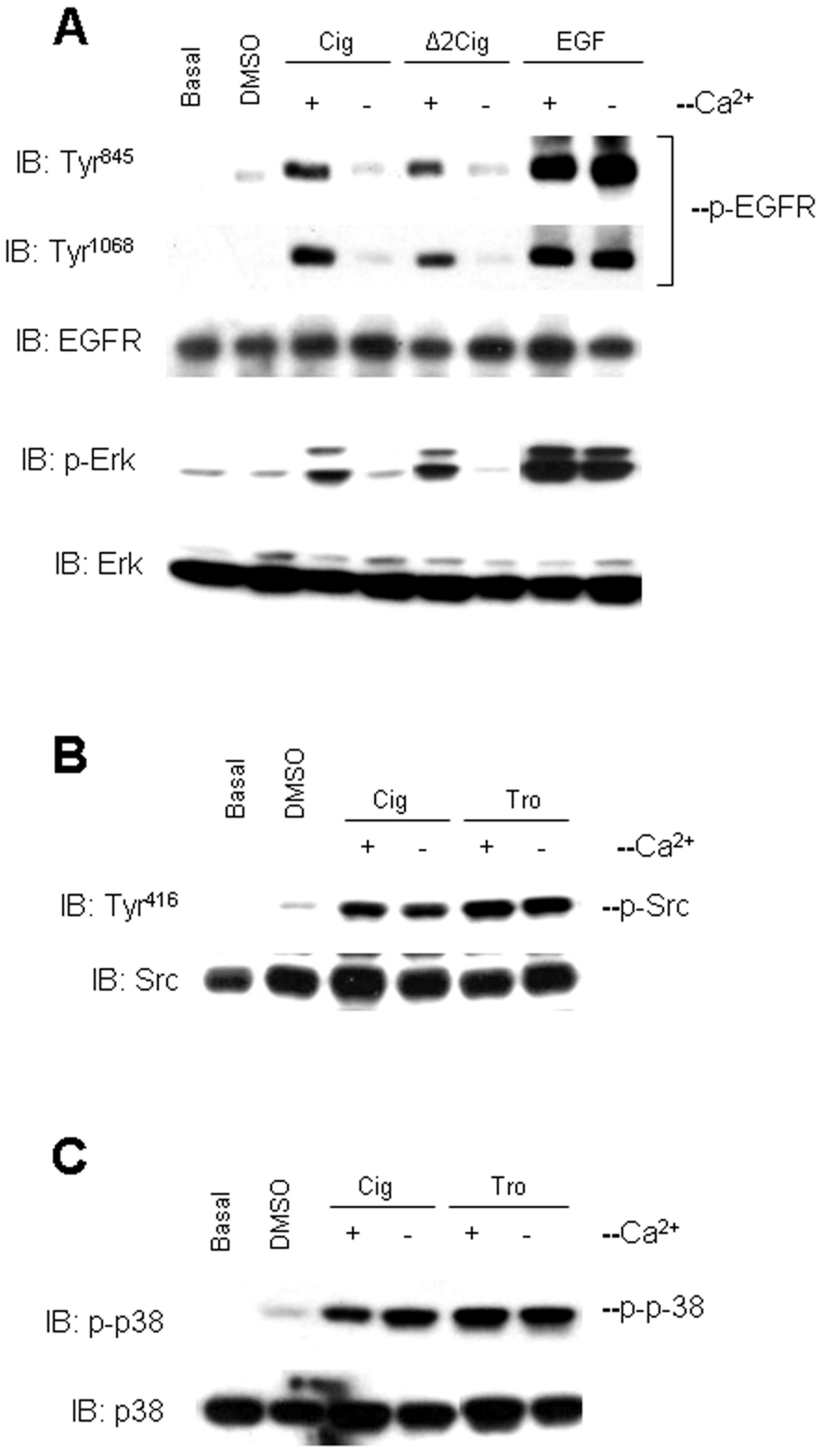

800
600
400
200
0
HBSS(+Ca2+)+TZD
HBSS (-Ca2+)
50 μM Cig
50 μM Tro
[Ca2+]i (nM)
0
200
400
600
800
1000
1200
Time (sec)

FIGURE 9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DMSO
Cig
Δ2Cig
EGF
+ - + - + -
--Ca2+
IB: Tyr845
IB: Tyr1068
--p-EGFR
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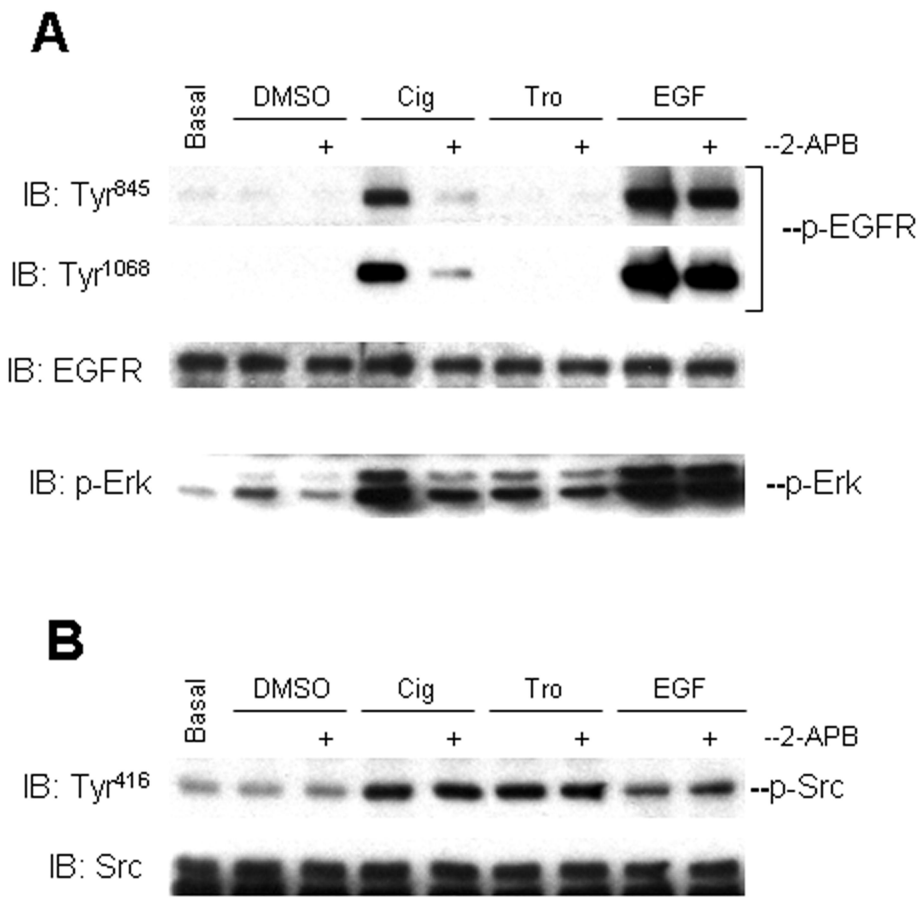
IB: p-Erk
IB: Erk
B
Basal
DMSO
Cig
Tro
+ - + -
--Ca2+
IB: Tyr416
--p-Src
IB: Src
C
Basal
DMSO
Cig
Tro
+ - + -
--Ca2+
IB: p-p38
--p-p-38
IB: p38

FIGURE 10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EGF
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+
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--2-APB
IB: Tyr845
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IB: p-Erk
--p-Erk
B
Basal
DMSO
Cig
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+
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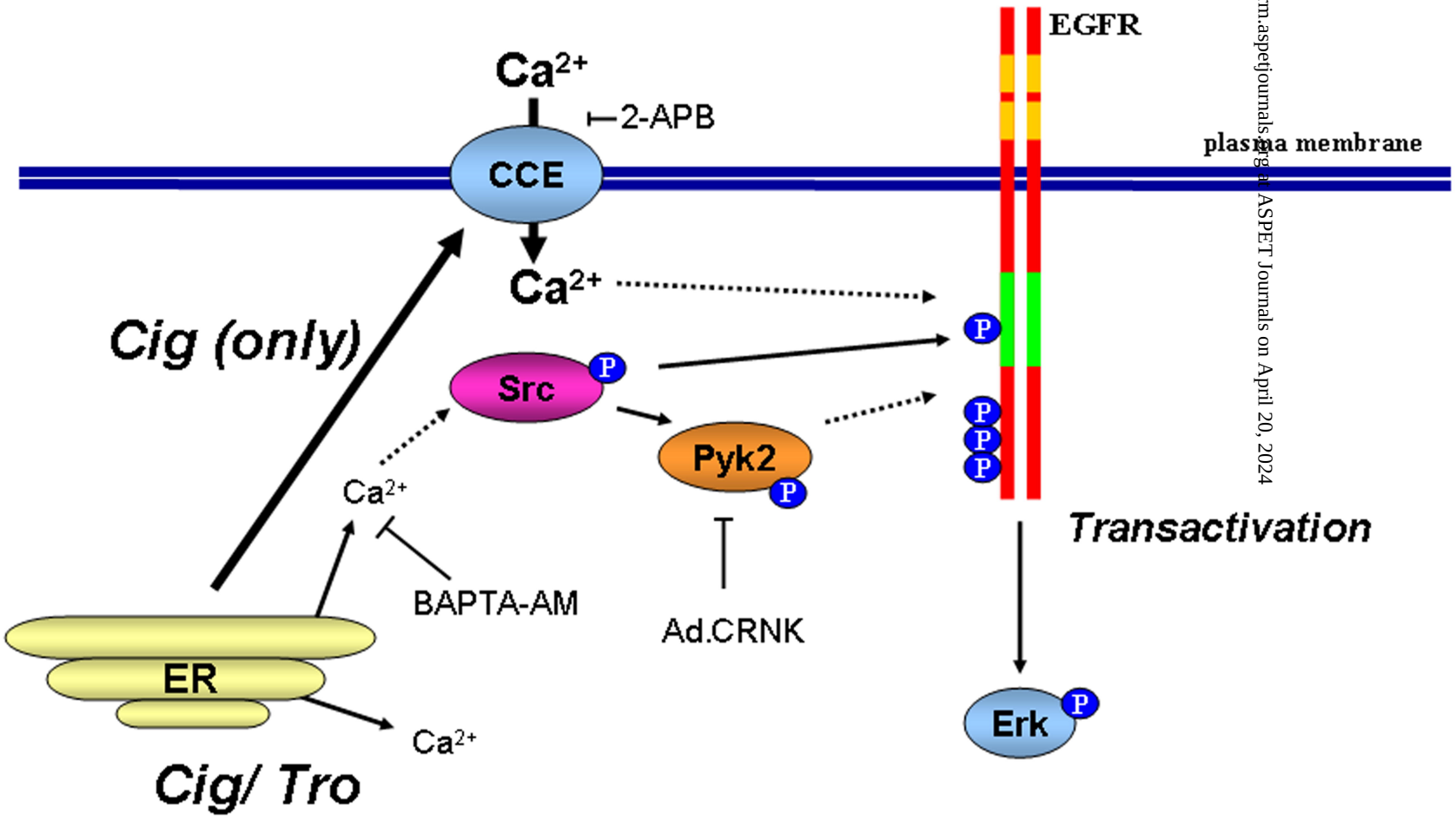
IB: Src

FIGURE 11



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