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Targeting of PKC ζ and PKB to caveolin-enriched microdomains represents a crucial step underpinning the disruption in PKB-directed signalling by ceramide

Eric Hajduch^{1,2†}, Sophie Turban^{3†}, Xavier Le Liepvre^{1,2}, Soazig Le Lay^{1,2}, Christopher Lipina³
Nikolaos Dimopoulos³, Isabelle Dugail^{1,2}, Harinder S Hundal^{3*}

¹INSERM, UMR S 872, Centre de Recherche des Cordeliers, Paris, F-75006, France

²Université Pierre et Marie Curie-Paris 6, UMR S 872, Paris, F-75006, France

³Division of Molecular Physiology, Sir James Black Centre, College of Life Sciences, University of Dundee, Dundee, DD1 5EH, United Kingdom

† These authors contributed equally to the work reported.

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Abbreviations: CEM, caveolin-enriched microdomains; PKC ζ , protein kinase c-zeta; PKB, protein kinase B; PIP₃, phosphoinositide 3,4,5 triphosphate; C2-Ceramide (N-acetylshingosine), M β CD, methyl β -cyclodextrin.

INSERM, UMR S 872, & Université Pierre et Marie Curie-Paris 6, Paris

Address correspondence to:

Dr Hari Hundal,
Division of Molecular Physiology,
Sir James Black Centre,
College of Life Sciences,
University of Dundee,
Dundee, DD1 5EH, UK.

Tel: (+44) 1382 384969;

Fax: (+44) 1382 385507;

E-mail: h.s.hundal@dundee.ac.uk

SYNOPSIS

Elevated ceramide concentrations in adipocytes and skeletal muscle impair PKB/Akt-directed insulin signalling to key hormonal end-points. An important feature of this inhibition involves the ceramide-induced activation of atypical PKC ζ , which associates with and negatively regulates PKB. Here we demonstrate that this inhibition is critically dependent upon the targeting and subsequent retention of PKC ζ -PKB within caveolin-enriched microdomains, which is facilitated by kinase interactions with caveolin. Ceramide also recruits PTEN, a 3'-phosphoinositide phosphatase, thereby creating a repressive membrane microenvironment from which PKB cannot signal. Disrupting the structural integrity of caveolae by cholesterol depletion prevented caveolar targeting of PKC ζ and PKB and suppressed kinase-caveolin association, but, importantly, also ameliorated ceramide-induced inhibition of PKB. Consistent with this, adipocytes from caveolin-1^(-/-) mice, which lack functional caveolae exhibit greater resistance to ceramide compared with caveolin-1^(+/+) adipocytes. We conclude that the recruitment and retention of PKB within caveolin-enriched microdomains contributes significantly to ceramide-induced inhibition of PKB-directed signalling.

INTRODUCTION

Impaired insulin action may develop as a result of defects at any point in the signal transduction process initiated by the activated insulin receptor kinase, which tyrosine phosphorylates intracellular target substrates, in particular the family of insulin receptor substrates (IRS proteins) [1]. Whilst numerous proteins can dock with activated IRS proteins there is general acceptance that the serine/lipid kinase phosphoinositide 3-kinase (PI3K) and signalling effectors downstream from it, in particular protein kinase B (PKB, also known as Akt), play a crucial role in glucose homeostasis. Mice lacking PKB β (Akt2), for example, become insulin resistant and develop severe diabetes [2] suggesting that normal activation of PKB and the fidelity with which it signals will have profound implications for many insulin-regulated events, including glycogen synthesis and glucose transport [3].

The activation of PKB by insulin relies upon its recruitment to the plasma membrane. Precisely how this occurs is unclear but the process is facilitated by the binding of 3-phosphoinositides (e.g. PIP₃) to the N-terminal pleckstrin homology (PH) domain of the kinase, which induces conformational changes in PKB [4] that expose its two regulatory phosphorylation sites, Thr³⁰⁸ and Ser⁴⁷³ (in PKB α). Phosphorylation of Thr³⁰⁸ is mediated by PDK1 whilst that of Ser⁴⁷³ is catalyzed by the mTOR-Rictor (TORC2) complex [5]. We, and others, have shown that insulin's ability to recruit and activate PKB at the plasma membrane is dramatically attenuated by over-supply of saturated fatty acids (e.g. palmitate) that promote intracellular synthesis of ceramide [6,7], a lipid whose accumulation in insulin target cells has been implicated strongly in the pathogenesis of insulin resistance. Indeed, a very recent study has elegantly identified ceramide as a key second messenger linking excess fatty acids to the induction of insulin resistance *in vivo* [8]. The loss in PKB activation induced by ceramide has been proposed to occur by two possible mechanisms, although these need not be mutually exclusive. The first involves activation by ceramide of a Type 2A-like phosphatase activity that promotes dephosphorylation of Thr³⁰⁸/Ser⁴⁷³ [9], whereas, the second involves a loss in membrane recruitment and phosphorylation of PKB [10] by a process dependent upon activation of atypical protein kinase C isoforms (PKC λ/ζ) [11,12]. PKC ζ directly interacts with and represses the activity of PKB in numerous cell types [11-16], but dissociates upon cell treatment with growth factors [13] and insulin [12] thereby allowing PKB to become activated. However, an increase in intracellular ceramide results in potent activation of PKC ζ , which stabilizes the interaction between the two kinases and, moreover, antagonises insulin's

ability to not only dissociate the kinase complex, but to also activate PKB [12]. We have established that the interaction between these two kinases requires the PKB-PH domain and that PKC ζ , when activated by ceramide, phosphorylates a threonine or serine residue (depending on the PKB isoform) at site 34 within this domain [12]. This phosphorylation suppresses PIP₃ binding to the PH-domain thus reducing activation of PKB at the plasma membrane [12].

Although ceramide can directly bind to and activate PKC ζ [17], such interactions *in vivo* are likely to involve recruitment of the kinase to ceramide-enriched membrane domains. Ceramide is a membrane lipid and it is widely acknowledged that detergent-resistant membrane (DRM) domains (typically enriched in cholesterol, sphingomyelin and sphingomyelinases), integrate the cholesterol-binding protein, caveolin, to form flask like invaginations (i.e. caveolae) that serve as foci for the production of highly localized concentrations of ceramide [18]. Indeed, ~50-70% of the cellular ceramide and sphingomyelin may be contained within caveolin-enriched membrane domains [19]. Caveolin is capable of forming scaffolds *via* a short stretch of its cytosolic N-terminal domain onto which signalling complexes can assemble [20]. This scaffold function may serve to functionally regulate the activation status of associated signalling proteins by either placing them in close proximity to other caveolar constituents or by sequestering them from regulatory proteins that may be excluded from caveolae. Caveolin can interact with PKC ζ *via* its scaffolding domain [21] and this may explain the propensity to accumulate PKCs [22] in caveolae where they would be susceptible to direct activation by ceramide contained within such domains [17]. These observations raise the intriguing possibility that a key aspect of ceramide induced insulin resistance may involve sequestration and repression of PKB, via its physical association with PKC ζ , in caveolin-enriched microdomains (CEM).

In this study, we have examined the potential involvement of CEM in facilitating the suppressive effects of ceramide on PKB-directed insulin signalling. We find that ceramide, but not insulin, induces recruitment of PKB and PKC ζ to caveolin-enriched DRMs and that under these circumstances association of PKC ζ with caveolin-1 is enhanced and activation of PKB in response to insulin is dramatically reduced. Importantly, we demonstrate that disrupting these microdomains by cholesterol depletion not only prevents recruitment of both kinases to DRMs, but also ameliorates the loss in PKB activation. Moreover, we present

evidence showing that ceramide's ability to impair PKB activation is considerably diminished in adipocytes of mice that are genetically deficient in caveolin-1 and which consequently lack functional caveolae.

EXPERIMENTAL

Materials - All reagent-grade chemicals, Protein A-Sepharose beads, insulin, M β CD, collagenase type II, methyl-isobutylmethylxanthine (IBMX), dexamethasone, BSA and palmitate were purchased from Sigma-Aldrich (Poole, U.K.). C2-ceramide was obtained from Tocris (Bristol, U.K.). Complete protein phosphatase inhibitor tablets were obtained from Boehringer-Roche Diagnostics. Ro-31-8220 was obtained from Calbiochem (San Diego, USA). Antibodies against caveolin-1, caveolin-3, and flotillin-1 were purchased from BD Biosciences (San Jose, USA), PKB and PKB-Ser⁴⁷³ were from Cell Signaling (New England Biolabs, Hertfordshire, UK), PKC ζ (Santa Cruz Biotechnology, USA), PTEN (Biosource, Germany), transferrin receptor (Hybrid Tech., USA). Horse-radish peroxidase (HRP)-anti rabbit, anti-mouse and anti-sheep/goat IgGs were from Jackson ImmunoResearch Laboratories (West Grove, USA) and the enhanced chemiluminescent substrate was from Pierce-Perbio Biotechnology (Germany).

Animal and Human tissue - Animal studies were conducted in accordance with French guidelines for the care and use of experimental animals. Caveolin-1 null mice used in this study were described elsewhere [23]. All experiments were performed on male mice from which epididymal fat pads were excised and used for isolating adipocytes by the method described by Rodbell [24]. Subcutaneous human fat was obtained from non-diabetic subjects who were undergoing elective abdominal surgery at the Department of General Surgery, Hospital Saint Louis, Paris. Samples were collected with the approval of the Paris Saint-Louis Ethics Committee and all subjects gave written consent. Subjects on endocrine therapy (e.g., steroids, HRT, thyroxine) or antihypertensive therapy and patients with malignant diseases were excluded. Human adipocytes were isolated from whole fat by the method described by Rodbell [24].

Culture of 3T3-L1 adipocytes and L6 muscle cells - 3T3-L1 cells were maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum. Adipocyte differentiation was then induced by adding IBMX (100 μ M), dexamethasone (0,25 μ M) and insulin (1 μ g/ μ l) for 2 days, and then cultured only in high-

glucose DMEM, 10% FCS for the duration of the differentiation period. Differentiated cells were usually harvested at day 10-12 following confluence. L6 rat skeletal muscle cells were grown as described previously [25] in α -minimal essential medium containing 2% (v/v) fetal bovine serum and 1% (v/v) antimycotic-antibiotic solution.

Preparation of whole cell and tissue lysates and plasma membranes – Human and 3T3-L1 adipocytes and L6 myotubes were lysed following experimental manipulation (see figure legends) in an appropriate volume of lysis buffer [25]. In some experiments, plasma membranes and cytosol from differentiated 3T3-L1 adipocytes and L6 myotubes were prepared by subcellular fractionation as previously described [25,26]. Protein content in whole cell/tissue lysates and membrane fractions was determined by the method of Bradford [27].

Preparation of detergent-resistant membranes (DRM) – DRM were prepared as described previously [28]. Cells were washed 3 times with ice-cold PBS and homogenized into 25mM MES (2-[N-Morpholino]ethanesulfonic acid), pH 6.0, 150mM NaCl, 1% (w/v) Triton X-100, complete inhibitor tablet. 2ml of lysate was diluted in 2ml of 80% (w/v) sucrose, 25mM MES, pH 6.0, 150mM NaCl. The gradient was then set up by adding 2ml each of 35%, 25%, 15% and 5% sucrose (w/v) in 25mM MES, pH 6.0, 150mM NaCl. The gradient was centrifuged at 120,000 g for 20h at 4°C in a SW41 Beckman rotor. DRM fractions (1ml) were then collected with a syringe and frozen at -20°C until required.

Immunoblotting - Cell lysates were subjected to SDS/PAGE on polyacrylamide gels and immunoblotted as previously reported [25]. Nitrocellulose membranes were probed with various antibodies described in the figure legends. Detection of primary antibodies were performed using appropriate peroxidase-conjugated IgGs and protein signals were visualized using enhanced chemiluminescence by exposure to Kodak autoradiographic film.

PKB and caveolin-1 immunoprecipitation from 3T3-L1 adipocyte lysates – 3T3-L1 adipocytes were extracted in 500 μ l lysis buffer and. PKB or caveolin-1 were immunoprecipitated from 100 μ g of cell lysates. Immunocomplexes were captured by incubation with protein A-agarose beads and solubilized in Laemmli sample buffer prior to SDS-PAGE and immunoblotting as described above.

Determination of cellular cholesterol content – Total lipids were extracted as described by Folch [29]. Dried lipids were resuspended in isopropanol, and determination of free cholesterol was performed using an F-CHOL (Roche Molecular Biochemicals) kit.

Analysis of cell viability – Subconfluent L6 myoblasts were incubated in the absence or presence for periods indicated in the figure legends with 100 μ M C2-ceramide, C2-ceramide/5mM M β CD, 0.5 mM palmitate or 0.5 mM palmitate and 10 μ M myriocin (a inhibitor of serine palmitoyl transferase which catalyses ceramide synthesis from palmitate). All incubations involving ceramide or palmitate were done in the presence of 0.2% (w/v) BSA as lipid conjugating protein. Viable cells were classified as those that were adherent, displayed trypan blue exclusion and which stained positively with 4,6-diamino-2-phenylindole diacetate (DAPI). Stained cells were visualized by using an Axiovert 200 fluorescence microscope and quantified by counting individual nuclei from several randomly chosen visual fields.

Statistical analysis - Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Newman-Keuls post test. Data were considered statistically significant at p values ≤ 0.05 and notified by *.

RESULTS

Effects of methyl β -cyclodextrin on membrane cholesterol content

Since it is well established that cholesterol is a fundamental component of CEM we postulated that if such microdomains participate in suppressing the hormonal activation of PKB in response to ceramide their disruption should antagonize the insulin-desensitizing effects of the sphingolipid. We initially monitored the effects of the cholesterol depleting agent, methyl β -cyclodextrin (M β CD), on membrane cholesterol content. The lipid composition of CEM facilitates their isolation as low density, detergent resistant membranes (DRM) on discontinuous sucrose density gradients, which can be subsequently assayed for cholesterol and protein content. Figure 1A shows that the abundance of cholesterol in isolated membrane fractions from 3T3-L1 adipocytes was greater in the low density DRM fractions, corresponding to those enriched in caveolins (peaking in fraction 5). Following cell treatment with 5mM M β CD there was a sizable reduction in the cholesterol content of this fraction. It should be stressed that previous work from our group [30] has shown that higher

concentrations of M β CD do not promote greater loss of membrane cholesterol and so all subsequent studies were performed using 5 mM M β CD. Analyses of caveolin-1 abundance revealed that DRM fractions harboring the greatest content of cholesterol were also those that were most enriched in caveolin-1 (Figure 1A). A similar analysis of membrane fractions isolated from L6 myotubes revealed the presence of cholesterol in two distinct regions of the sucrose gradient, both of which were also enriched with caveolin-3 (the muscle specific isoform, Figure 1B). It is likely that caveolin-3 present in the more dense detergent soluble fractions represents a pool that resides in non-caveolar membranes which have been described in some cell types [31]. This latter proposition is supported by the finding that the transferrin receptor (TfR), an archetypal non-lipid raft marker, populates the more dense membrane fractions isolated from both muscle and fat cells. Although 70 to 80% of exogenously added short-chain ceramide has been shown to accumulate within structured caveolin membrane microdomains [32], incubation of adipocytes or muscle cells with C2-ceramide had no detectable effect upon the distribution of either caveolin isoform in the gradient indicating that the lipid *per se* does not appear to affect the integrity or the buoyant density of CEM (Figure 1A, B). It is noteworthy that for either cell type the protein content along the gradient was “bottom heavy” with more than 95% of the protein localizing to the more dense membrane fractions (fractions 9 to 12) that comprise predominantly the cytosolic and detergent-soluble proteins.

Consistent with the reduction in DRM cholesterol content observed for both 3T3-L1 adipocytes and L6 myotubes in response to M β CD treatment, the abundance of the respective caveolin proteins expressed in these two cell types fell in DRMs and, as such, both were found to be dispersed in fractions harvested along the entire gradient. This dispersion arises as a result of a loss in the structural integrity of membrane caveolae given that in separate immunogold electron microscopy-based experiments (see supplementary data), we find that the labeling of caveolin-1 that is normally associated with “bulb” like membrane invaginations is reduced significantly (by ~70%) upon cholesterol depletion of 3T3-L1 adipocytes. Consistent with these findings we also observe, using scanning electron microscopy (see supplementary data), that cell treatment with M β CD induces a qualitative reduction in the number of membrane pits (of the reported size (~ 20 nm) of caveolar openings [33]) on the extracellular surface of 3T3-L1 adipocytes.

Cholesterol depletion antagonises the ceramide-induced loss in PKB activation.

The upper panel of Figure 2A shows that treatment of L6 myotubes with C2-ceramide alone has no detectable effect on PKB phosphorylation. However, in line with previous work [6,12], preincubation of differentiated 3T3-L1 adipocytes or L6 myotubes with 100 μ M C2-ceramide led to a significant (up to 80%) reduction in insulin's ability to activate PKB based on phosphorylation of Ser⁴⁷³ (Figure 2A, similar results were obtained using a phospho-antibody directed against PKB-Thr³⁰⁸, data not shown). This loss in phosphorylation was not observed in response to an equivalent concentration of C2-dihydroceramide, a structurally related molecule used here as a control, and was significantly attenuated in both fat and muscle cells depleted of cholesterol by the use of M β CD, during the latter 30 minute incubation of cells with ceramide. Intriguingly, if cells were incubated with M β CD that had been pre-complexed with cholesterol (M β CD/Chol), an intervention that we have previously shown to nullify its capacity to subsequently deplete membrane cholesterol [30], ceramide retains its ability to suppress the hormonal activation of PKB in both adipocytes and L6 myotubes (Figure 2A). This latter finding underscores the importance of cholesterol-enriched membrane domains in supporting ceramide action and, moreover, excludes the possibility that the ability to antagonise PKB activation in cells treated with M β CD is related to an effect of the compound other than loss in membrane cholesterol.

The availability of human adipose tissue allowed us to assess whether the findings made in two established insulin-responsive clonal cell lines extend to freshly isolated human adipocytes. Figure 2B shows that an acute insulin challenge led to a clear activation of PKB in human adipocytes, which was absent in adipocytes pretreated with C2-ceramide. Consistent with previous work in cultured L6 cells and 3T3-L1 adipocytes [6,12] showing that this impaired activation of PKB involves atypical PKCs, co-treatment of human adipocytes with ceramide and Ro 31.8220 (a compound that inhibits atypical PKCs when used at low micromolar concentrations) antagonised the ceramide-induced loss in PKB activation. This antagonism was not observed at submicromolar concentrations of the inhibitor which is known to suppress the activity of novel and conventional PKC isoforms (data not shown). Notwithstanding any selectivity issues that may be associated with use of Ro 31.8220, it is important to point out that we have previously substantiated the involvement of PKC ζ in the ceramide-induced inhibition of PKB by a number of different approaches [12] and that the

ability of Ro 31.8220 to counter the suppressive effect of ceramide on PKB in human adipocytes is consistent with the suggestion that PKC ζ is involved. Furthermore, in line with the data presented in Figure 2A, the observed loss in PKB activation in human adipocytes was significantly blunted in cells that had been cholesterol-depleted by M β CD prior to incubation with ceramide (Figure 2C). Lysates prepared from human adipocytes were also immunoblotted using phospho-antibodies against AS160 (Thr⁶⁴²), a physiological PKB target, and the PAS antibody which recognizes proteins phosphorylated on a consensus PKB motif. In line with the effects of ceramide on PKB activation, the insulin-dependent phosphorylation of AS160 at Thr⁶⁴² and that of other PKB substrates was reduced by the lipid and rescued in cells that had been cholesterol depleted using M β CD (Figure 2C). The above findings suggest that ceramide is likely to impair insulin action, at least at the level of PKB, by a common mechanism in all three cell types.

Differential effects of ceramide and insulin on PKC ζ and PKB distribution in DRMs and plasma membrane.

We and others have reported that PKC ζ can interact with and negatively regulate the activity of PKB in response to ceramide [11-13]. Since caveolin can interact with PKC ζ [21] and cholesterol depletion antagonizes the ceramide-induced loss in PKB activation we entertained the possibility that the kinase may be targeted to CEM by ceramide. To assess this possibility DRMs isolated from 3T3-L1 and human adipocytes as well as from L6 myotubes were immunoblotted using PKC ζ antibodies. Figures 3A, B and C show that PKC ζ was detected in the protein-enriched detergent soluble fractions (i.e. fractions 9-12) isolated from all three cell types. While ceramide treatment did not promote any gross shift in protein distribution in the gradient (data not shown) there was a clear increase in PKC ζ immunoreactivity in the low density DRM fractions from all three cell types. Cholesterol depletion *per se* had no detectable effect on the distribution of PKC ζ in adipocytes or L6 myotubes, but repressed the increase of the kinase in DRMs upon ceramide treatment. We subsequently performed a more focused analyses of the effects of ceramide and insulin on PKC ζ and PKB abundance in isolated DRM fractions from 3T3-L1 adipocytes (i.e. fractions 4-6). Figure 3D shows that whilst both kinases were detectable in DRM fractions from unstimulated adipocytes their abundance was elevated by more than 2-fold in fraction 5 following treatment of cells with ceramide or when adipocytes were simultaneously exposed to insulin and ceramide (Figure 3D). Insulin alone had no detectable effect on the abundance of either kinase in this DRM

fraction and so we attribute the increased presence of both proteins in this fraction to ceramide, which we did not observe in cholesterol depleted adipocytes (Figure 3D). Similar observations were also made in both L6 myotubes and human adipocytes (data not shown). It is noteworthy, that whilst insulin did not promote any net gain of PKB in the DRM fractions the hormone clearly increased its abundance in plasma membranes prepared from adipocytes by subcellular fractionation (Figure 3E). Given that isolated plasma membranes are biochemically and compositionally distinct from DRMs based on detergent solubility and caveolin-1 content (note the DRM fraction harbors ~4-fold more caveolin-1 when equivalent amounts of protein from both membrane fractions were immunoblotted, Figure 3E), our findings indicate that whilst activation of PKB requires its recruitment to the cell surface [34], it is likely to be targeted primarily to non-caveolar regions of the plasma membrane. In addition, Figure 3E shows that insulin does not induce any visible decrease in cytosolic PKB content, suggesting that only a small amount of PKB is translocated to the plasma membrane in response to the hormone.

Ceramide is a potent activator of PKC ζ [12,17] and suppressing its activation using Ro 31.8220 or bisindolymaleimides has been shown to significantly ameliorate the attendant loss in PKB activation [6,12] (Figure 2B). Interestingly, therefore, analysis of DRM fractions prepared from adipocytes treated with ceramide and 5 μ M Ro 31.8220 revealed that relative to flotillin-1, another DRM marker protein, the inhibitor suppressed the gain of PKC ζ in DRM by ceramide (Figure 3F). This finding implies that activation of PKC ζ may be an important requirement for its recruitment and/or retention in CEM and for its ability to prevent the hormonal activation of PKB in response to ceramide.

To further substantiate the physiological importance of the observed recruitment of PKC ζ to CEM in response to cell permeant analogues of ceramide we also assessed whether this recruitment was induced in response to prolonged incubation of L6 myotubes with the saturated fatty acid, palmitate (Figure 3G). Palmitate can be utilized for the *de novo* synthesis of ceramide by pathway that is dependent on the rate-limiting enzyme serine palmitoyl transferase (SPT). Long chain (C16) ceramides generated from palmitate *via* the SPT pathway promote a loss in PKB-directed insulin signalling by the same mechanism that we have detailed for short chain ceramide analogues [6]. Figure 3G shows that low density DRM-containing fractions isolated from L6 myotubes exhibit increased PKC ζ immunoreactivity following cell incubation with palmitate for 16h. This immunoreactivity was noticeably

reduced in DRM fractions isolated from myotubes that had been coincubated with palmitate and the SPT inhibitor myriocin, which effectively suppresses palmitate-driven ceramide synthesis [6].

Effects of insulin and ceramide on PTEN abundance in DRMs

Recent studies in neurotumour cells have shown that the tumour suppressor PTEN is recruited to lipid rafts by ceramide where it may facilitate a reduction in 3'phosphoinositides and thereby prevent activation of PKB at these membrane loci [35]. To assess whether this also occurs in 3T3-L1 adipocytes we immunoblotted membrane fractions with anti-PTEN antibodies. Figure 4 shows that compared to untreated adipocytes, PTEN abundance was noticeably elevated by over 2-fold in caveolin-enriched DRM fractions following ceramide treatment. In contrast, insulin had no significant effect on PTEN content in adipocyte DRMs.

The effects of ceramide and cholesterol depletion upon interactions between PKC ζ , PKB and caveolin-1

In line with previous work from our lab [12] and that of many others [11,13-15] immunoprecipitation of PKB from unstimulated cells coprecipitated PKC ζ (Figure 5A). This interaction was enhanced in response to ceramide (1.8-fold) consistent with the idea that PKC ζ acts to negatively regulate PKB *via* this association [13], whereas insulin reduced this interaction to a level where the immunoreactive PKC ζ signal in PKB immunoprecipitates was comparable to that seen in the control IgG lane (Figure 5A). The increased association seen following treatment of adipocytes with ceramide was not observed in cholesterol depleted cells (Figure 5A). It is noteworthy that whilst M β CB antagonized the ceramide-induced association of PKC ζ and PKB this antagonism was not observed if cells were incubated M β CB that had been precomplexed with cholesterol (Figure 5A lower panel). These findings imply that the interaction between PKC ζ and PKB in response to ceramide requires a cholesterol-enriched microdomain. Of interest, however, was the finding that caveolin-1 also coprecipitates with PKB following cell incubation with ceramide, and this too was reduced in cholesterol depleted cells (Figure 5A upper panel). In comparison to ceramide, insulin did not promote any significant association of PKB with caveolin-1 (Figure 5A). We subsequently performed caveolin-1 immunoprecipitations and probed the immunopellet with antibodies to PKC ζ . This approach revealed that association of caveolin-1 with PKC ζ was enhanced by more than 2-fold in cells incubated with ceramide (Figure 5B), whereas this interaction was

antagonized by treating cells with M β CD. It proved difficult to establish in these experiments whether PKB also coprecipitated with caveolin-1 as we were unable to satisfactorily resolve the immunoreactive PKB signal from the very closely migrating, and much more prominent, IgG heavy chain signal (data not shown). It should also be stressed that given the very low protein recovery it was not feasible to perform immunoprecipitation of PKB or PKC ζ from DRM fractions.

Insights from caveolin-1 deficient mice

To help further define the role of CEM with respect to ceramide action on PKC ζ and PKB activation we studied adipocytes from transgenic mice lacking functional caveolae domains owing to a genetic deficiency in caveolin-1 [23]. Adipocytes isolated from wild-type mice revealed that whilst we were able to detect weak association of PKC ζ with PKB (based on coprecipitation analysis) in unstimulated cells, ceramide significantly increased the association between the two kinases by over 3-fold (Figure 6A). In contrast, even after prolonged exposure of the autoradiogram shown we were unable to detect any increase in interaction between the two kinases following incubation of caveolin-1 deficient adipocytes with ceramide. We subsequently used adipocytes from control and caveolin-1 knock-out mice to assess the effects of ceramide on the hormonal activation of PKB. Figure 6B shows that insulin caused a robust activation/phosphorylation of PKB in wild type adipocytes that fails to occur if cells were preincubated with ceramide. In contrast, adipocytes lacking caveolin-1 retain a significant capacity to permit activation of PKB despite having been incubated with ceramide. This latter finding implies that a deficiency in caveolin-1 and hence caveolae confers an element of resistance to the insulin-desensitizing effects of ceramide.

Cholesterol depletion antagonizes ceramide-induced cell death

In addition to promoting insulin resistance, ceramide is known to also induce cell death. However, it should be stressed that our studies utilizing muscle and fat cells were all performed in their fully differentiated state. We have previously reported that because L6 myoblasts exhibit much greater cell cycle activity they are far more susceptible to ceramide-induced death than terminally differentiated myotubes [12]. Nevertheless, we postulated that since cholesterol depletion antagonizes ceramide signalling (known to promote cell death) and reinstates activation of PKB (a prosurvival signal) that this manipulation may help improve the viability of L6 myoblasts when challenged with ceramide. Consistent with this

expectation, L6 myoblasts that were cholesterol depleted just prior to incubation with ceramide exhibited better rates of survival based on trypan blue exclusion and DAPI staining (Figure 7).

DISCUSSION

The findings presented here collectively suggest that in conjunction with atypical PKC ζ , CEM play a critical part in the mechanism by which ceramide impairs the hormonal activation of PKB in adipocytes and cultured rat skeletal muscle cells. The lipid composition of caveolae is considered distinct from the planar membrane domain and is particularly enriched with cholesterol, phosphatidylinositols and sphingolipids (sphingomyelin and glycosphingolipids) [36,37]. The presence of sphingomyelin and sphingomyelinases within caveolae gives considerable scope for the highly localized production and accumulation of ceramide within these membrane microdomains [18,19]. Based on the data presented here and that in previous work [12], we propose a model (Figure 8) in which an increase in caveolar ceramide recruits PKC ζ in complex with PKB to CEM, where activation of PKC ζ not only stabilizes the kinase complex but also enhances its ability to negatively regulate PKB. Crucially, we suggest that sequestering both kinases within these membrane domains is critical for maintaining PKB in its repressed state and that, as such, recruitment and retention involves interaction of the kinase complex with caveolin-1. Whilst we were unable to demonstrate a direct physical association between PKC ζ and PKB within CEM, the observed coprecipitation of PKC ζ and caveolin-1 in PKB immunoprecipitates from ceramide-treated cells (Figure 5A) implies that caveolin-1 may, via its scaffolding domain [21], act to bridge the interaction between the two kinases in CEM. Consistent with studies in neurotumor (HOG and PC12 cells) cells [35], we also observe that ceramide recruits PTEN to caveolin-enriched DRMs of 3T3-L1 adipocytes. An important consequence of PTEN recruitment to DRMs would be diminished localized production of 3'-phosphoinositides, which, coupled with the inhibitory influence of PKC ζ [12], would create a repressive membrane microenvironment for PKB. Such repression is unlikely to extend to the bulk planar membrane given that we [10], and others [38], have shown that whilst ceramide inhibits PKB recruitment to the plasma membrane it does not suppress ligand (insulin or PDGF) stimulated synthesis of 3'-phosphoinositides. We suggest that ceramide's ability to recruit the pool of PKB normally activated by insulin to CEM spatially segregates the kinase from those membrane regions that remain competent for 3'-

phosphoinositide synthesis, and which otherwise would be capable of supporting the hormonal activation of PKB. It is noteworthy that our findings are broadly consistent with recent work from Kester's group [32], which appeared whilst this manuscript was in preparation. Fox *et al* have also observed that ceramide recruits PKC ζ to structured microdomains in vascular smooth muscle cells and that this is linked with the lipid's ability to not only promote inhibition of PKB/Akt activation but to also induce cellular growth arrest [32]. Our findings suggest that in addition to vascular smooth muscle cells the ceramide-induced recruitment of PKC ζ to structured microdomains and the associated inhibition in PKB-directed signalling is also mechanistically preserved in both cultured and primary adipocytes from mice and humans as well as in cultured rat skeletal muscle cells. We propose that targeting of PKC ζ and PKB to CEM is likely to represent an important step in mediating the suppressive effects of ceramide on PKB signalling and cell viability. The observation that disrupting the structural integrity of caveolae alleviates the repressive effects of ceramide on cell viability, PKB activation and that adipocytes deficient in functional caveolae retain a significant capacity to stimulate the kinase in response to insulin in the presence of ceramide would strongly support this supposition.

The finding that activation of PKC ζ by ceramide antagonizes insulin signalling may seem incongruous with reports in the literature showing that activation of this kinase supports the anabolic effects of insulin to key end points such as glucose transport [39]. However, there is growing recognition that functional specificity of atypical PKC signalling may rely upon the kinase forming interactions with different protein regulators and adapters and that such interactions may be regulated in a stimulus-dependent manner [40]. We propose that the interaction between PKC ζ and PKB serves as one example of how this functional specificity in signalling is achieved. In unstimulated cells, the interaction between the two proteins serves to repress PKB [13], but upon cell stimulation with PDGF or insulin the kinase complex dissociates allowing both proteins to be activated and to participate in growth factor/insulin signalling [12,13]. However, ceramide over-rides the insulin signal and suppresses dissociation of the kinase complex [12]. We would argue that this "over-ride" is critically dependent upon targeting the kinase complex to caveolae where it sequestered as a result of interactions formed therein between PKC ζ and caveolin. Localization of PKC ζ to CEM may also facilitate recruitment and/or interaction with other proteins participating in the control of insulin sensitivity. In this regard, the finding that palmitate induces recruitment of

PKC ζ to CEM is, we believe, of physiological significance. Members of the Toll-like receptor family (TLR2 and TLR4) have been implicated in fatty-acid induced insulin resistance [41,42] and, intriguingly, in the case of TLR4, pro-inflammatory stimuli induce its recruitment to lipid rafts in a PKC ζ -dependent manner [43], which may be an important factor that contributes to the pathogenesis of insulin resistance by inflammatory pathways. Consequently, depending on the nature of the stimulus and the protein interactions that PKC ζ may form, the kinase can act as molecular switch that either promotes or suppresses activation of signalling pathways affecting control of diverse cellular responses [40].

A key feature of the mechanism by which insulin/growth factors activate PKB involves recruitment of the kinase from the cytosol to the cell surface where it undergoes phosphorylation by its two upstream kinases [34]. Whilst this increase is detectable in plasma membranes isolated by subcellular fractionation [10], we did not observe any loss in cytosolic PKB content in response to insulin (figure 3E). This finding is in line with previous work from Hemming's lab who showed by quantitative immunogold electron microscopy that only a very small fraction (<5%) of the total PKB appears to be translocated to the cell surface for activation [34]. We suggest that this small fraction, which normally participates in insulin-dependent signalling, is targeted to CEM in response to ceramide (Figure 3D) where it is retained in a repressed state. The EM studies by Andjelkovic *et al* involved analysis of HA-tagged PKB α in 293 cells because antibodies against the native kinase were not useful for immuno-EM microscopy [34]. We have also found that whilst the commercial PKB antibodies used here work well for immunoblotting and immunoprecipitation they fail to label cell material that has been fixed for EM analysis. Consequently, this precluded use of immuno-EM to assess caveolar localization of PKB. Nevertheless, given that we see no enrichment of PKB in DRMs in response to insulin and find that the kinase is capable of being stimulated in response to the hormone in cholesterol depleted cells (Fig 2A) it would seem highly unlikely that insulin targets PKB to CEM for activation.

There remains some degree of contestation as to the importance of caveolae with respect to initiation of trans-membrane insulin signalling. Some investigators have suggested that insulin receptors segregate within caveolae and that cholesterol depletion markedly reduces downstream insulin signalling in rat adipocytes [44], whereas others have questioned caveolae involvement in insulin receptor signalling [45]. More recently, a very elegant electron microscopy-based study demonstrated that insulin receptors segregate at the necks, but not the

bulb, of caveolae [46]. We would suggest that whilst cholesterol depletion would disrupt the structural integrity of the invaginated “bulb” that insulin receptors normally resident at the neck of such structures are likely to redistribute within the planar membrane domain, but still retain their functional signalling capacity. If so, how is the impaired insulin-signalling (at the level of IRS1 and PKB phosphorylation) in cholesterol depleted rat adipocytes [44] and in adipose tissue of caveolin-1 deficient mice [47] reconciled? The concentration of M β CD used in the present study (5mM) was based on previous work in which we had optimized the most effective dose at which membrane cholesterol content was reduced [30]. It is conceivable that higher doses of M β CD (e.g. 10mM), as used in the study by Parpal and coworkers [44], may introduce effects on insulin signalling that are unrelated to changes in membrane cholesterol content. This possibility is supported by our finding that whilst raising the M β CD concentration from 5mM to 10mM promotes a loss in insulin-stimulated PKB phosphorylation in 3T3-L1 adipocytes, it does so in the absence of any further reduction in membrane cholesterol [30]. In contrast, the reduced insulin signalling potential reported in adipose tissue of caveolin-1 null mice is due to a significant deficit in insulin receptor abundance, which is brought about by its enhanced proteosomal degradation [47]. Caveolin interacts with the insulin receptor *via* its scaffolding domain and it is likely that this may serve to not only anchor receptors at the necks of caveolae, but possibly protect them from internalization and breakdown. In the absence of functional caveolae, a loss in insulin receptor copy would be expected to reduce insulin signalling; the lower insulin-dependent phosphorylation of PKB observed in adipocytes deficient in caveolin, *vis-à-vis* that seen in wild type adipocytes, is consistent with this expectation (Figure 6B). Nevertheless, despite this diminished signalling capacity, the finding that caveolin-deficient adipocytes exhibit greater resistance to ceramide than adipocytes from wild type mice, based on using PKB phosphorylation as a readout, substantiates the importance of functional caveolae in ceramide-induced insulin resistance.

In addition to suppressing insulin signalling, ceramide is also known to exert a negative effect on cell growth and survival [48]. We postulated that since PKB signalling plays a pivotal role in mediating some of the metabolic effects of insulin [2] and is a key determinant of cell survival and proliferation [49] that inhibition of PKB by ceramide may be an important factor contributing to cell death. Indeed, evidence supporting this proposition exists in the literature [50,51] and, accordingly, one expectation, based on the findings presented in this study, would

be that cholesterol depletion would antagonize the lipid's ability to promote cell death. Consistent with this view, we did observe that cell death following treatment with ceramide was reduced significantly (from 64% to 33%) upon coincubating myoblasts with ceramide and M β CD, the cholesterol depleting agent (Figure 7). These observations support the idea that ceramide-induced inhibition of PKB within CEM may, in part, be responsible for reduced cell survival. Indeed, the finding that expression of a ceramide-resistant PKB mutant (T34A) in L6 myoblasts antagonizes ceramide-induced cell death [12], whereas expression of PKB mutant (S34E) that mimics the "ceramide-sensitive" form of the kinase in a smooth muscle cell line [32] attenuates cell growth and proliferation is fully in keeping with this view. However, it is also likely that disrupting the integrity of caveolae-rafts by cholesterol depletion suppresses the activation of pro-death signals [52,53], which, in combination with a rescue in PKB signalling, contribute to the increase in cell survival. Consequently, greater understanding of how these membrane microdomains influence the localization and activity of signalling proteins such as PKC ζ and PKB is likely to be of significant value in designing strategies that not only help improve insulin sensitivity, but control cellular growth and survival.

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Figure Legends

Figure 1 : Fractionation and purification of detergent resistant membrane (DRM) fractions from 3T3-L1 adipocytes and L6 myotubes. 3T3-L1 adipocytes and L6 myotubes were treated with 100 μ M C2-ceramide for 2h or 5mM M β CD for 30 min before being solubilised in 1% Triton X-100 at 4°C and fractionated on sucrose gradients as described in the methods section. Fractions from (A) 3T3-L1 adipocytes and (B) L6 myotubes were collected from top to bottom of the gradient. An equal volume of each sucrose gradient fraction was subjected to SDS-PAGE and immunoblotted with antibodies against caveolin-1, caveolin-3 and the transferrin receptor (TfR). Cholesterol content (left ordinate) and protein concentration (right ordinate) were assessed in DRM fractions from (A) 3T3-L1 adipocytes and (B) L6 myotubes. Representative immunoblots from 3 independent experiments are shown.

Figure 2: Effects of cholesterol depletion and ceramide on PKB activation in adipocytes and muscle cells. (A) 3T3-L1 adipocytes and L6 myotubes were pre-incubated with 100 μ M C2-dihydroceramide (Dcer), or for 2h with 100 μ M C2-ceramide and/or 5mM M β CD or 5mM M β CD that had been pre-complexed with cholesterol (M β CD/Chol) that was added to cells during the last 30 min. Cells were then incubated with 100nM insulin for 10min before being lysed and immunoblotted with either a phospho-specific antibody directed against PKB Ser⁴⁷³ or a pan PKB antibody. The bar graphs show densitometric quantification of the phospho-PKB blots. (B, C) Isolated human adipocytes were incubated as described above and in some experiments, cells were pre-incubated with 5 μ M Ro 31.8220 for 30min prior to incubation with ceramide. At the end of this incubation period, 100nM insulin was added for a further 10min and cells subsequently lysed. Cells lysates were immunoblotted with antibodies against PKB Ser⁴⁷³, native PKB, AS160 Thr⁶⁴² or Phospho-(Ser/Thr) Akt Substrate (PAS) antibodies. Representative immunoblots from at least 3 independent experiments are shown.

Figure 3: Ceramide promotes targeting of PKC ζ and PKB to DRM fractions of adipocytes and L6 myotubes. 3T3-L1 adipocytes (A), human adipocytes (B), and L6 myotubes (C) were treated with 100 μ M C2-dihydroceramide (Dcer), C2-ceramide, for 2h and 5mM M β CD was added during the last 30min. Cells were then incubated with 100nM insulin for 10min before isolation of DRMs as described in methods. 1 μ g protein of each fraction was

then immunoblotted for the presence of PKC ζ . These are representative immunoblots from at least three independent experiments. (D) 3T3-L1 adipocytes were pre-incubated with 100 μ M C2-ceramide for 2h and with 5mM M β CD added during the last 30min. Cells were then incubated with 100nM insulin for 10min before being lysed and DRM fractions isolated. DRM-containing fractions 4 to 6 were then immunoblotted for the presence of PKC ζ , PKB and caveolin-1. Bands were quantified by scanning densitometry and expressed as fold increase over control. Each point is the mean \pm SEM of three independent experiments. (E) 3T3-L1 adipocytes were incubated with 100nM insulin for 10min prior to plasma membrane (PM), cytosol, and detergent resistant membrane (DRM) isolation. 1 μ g protein of DRM-containing fraction 5, isolated PM and cytosol fractions were immunoblotted with PKB and caveolin-1 antibodies. (F) 3T3-L1 adipocytes were treated with 5 μ M Ro 31.8220 for 30 min prior to incubation with 100 μ M C2-ceramide for 2h. Cells were then incubated with 100nM insulin for 10min before isolation of detergent resistant membranes as described above. 1 μ g protein of DRM-containing fractions 4 to 6 were then immunoblotted using antibodies against PKC ζ and flotillin-1. (G) L6 myotubes were incubated in the absence or presence of palmitate (0.75 mM that had been conjugated to BSA 0.2% (w/v)) for 16h. In some experiments fatty acid incubation was also done in the presence of 10 μ M myriocin. DRM fractions were isolated and immunoblotted with antibodies against PKC ζ or caveolin-3.

Figure 4: Ceramide promotes redistribution of PTEN to DRM fractions of 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with either 100nM insulin for 10min or 100 μ M ceramide for 2h prior to isolation of DRMs. 1 μ g of each fraction was immunoblotted for the presence of PTEN and caveolin-1. The blots shown are representative of three separate experiments. Scanning densitometry was performed to quantitate changes in PTEN abundance in the DRM fractions. Bars represent mean \pm SEM, the asterisk signifies a significant change $P < 0.05$ relative to the untreated control.

Figure 5: Ceramide promotes both PKC ζ /PKB and caveolin-1/PKC ζ interaction in 3T3-L1 adipocytes.

3T3-L1 adipocytes were treated with 100 μ M C2-ceramide for 2h and 5mM M β CD or 5mM M β CD that had been preloaded with Cholesterol (M β CD/Chol, lower panel), which was added during the last 30min. Cells were then incubated with 100nM insulin for 10min and lysed prior to immunoprecipitation of (A) PKB or (B) caveolin-1 (cav-1). PKB and Cav-1

immunoprecipitates were immunoblotted for the presence of PKC ζ , caveolin-1 and PKB. The bar graph represents data (mean \pm SEM) quantifying the interaction between PKB and PKC ζ from three independent experiments, the asterisk signifies a significant change ($P < 0.05$ relative to an untreated cells).

Figure 6: Adipocytes from caveolin-1 deficient mice exhibit greater resistance to ceramide compared with caveolin-1^(+/+) adipocytes.

(A) Caveolin-1^(+/+) and caveolin-1^(-/-) adipocytes were treated with 100 μ M C2-ceramide for 2h prior to being lysed and immunoprecipitation of PKB and immunoblotting of immunoprecipitates with antibodies against PKC ζ and PKB. Bands were quantified by scanning densitometry. (B) Caveolin-1^(+/+) and caveolin-1^(-/-) adipocytes were treated with 100 μ M C2-ceramide for 2h and incubated with 100nM insulin for a further 10min prior to be lysed. 10 μ g of cell lysate was immunoblotted with antibodies directed against PKB Ser⁴⁷³, native PKB and caveolin-1. Bands were quantified by scanning densitometry and expressed as fold change relative to the insulin treated cells. Bars in panels A and B represent the mean \pm SEM of three independent experiments, the asterisk signifies a significant change ($P < 0.05$ relative to the untreated wild type control).

Figure 7 - Effects of ceramide and cholesterol depletion on viability of L6 myoblasts:

To assess cell viability in response to ceramide and cholesterol depletion, sub-confluent L6 myoblasts were incubated in the absence or presence of 100 μ M ceramide (for 2h) and/or treated with 5 mM Methyl β -cyclodextrin during the last hour. Viable cells were classed as those that were adherent, displayed trypan blue exclusion and which stained positively with 4,6-diamino-2-phenylindole diacetate (DAPI). Stained cells were visualized using an Axiovert 200 fluorescence microscope and quantified by counting individual nuclei from 5 randomly chosen visual fields. (Values are Mean \pm SEM, asterisk represent a significant loss in cell number relative to the untreated sample, whereas the cross is a significant change between the two bars indicated, $P < 0.05$).

Figure 8: Proposed model explaining the loss in PKB-directed insulin signalling in response to an increase in caveolar membrane ceramide content. (A) Binding of insulin to its receptor at caveolar necks promotes activation of PKB and PKC ζ via tyrosine phosphorylation of IRS proteins and activation of PI3K. The concomitant increase in PIP3

facilitates PKB recruitment to the plasma membrane where it is activated by its upstream kinases PDK1 and TORC2 (not shown). PKB activation promotes stimulation of key end-point responses to insulin action such as glucose transport and glycogen synthesis; kinase activation also provides an important growth and prosurvival signal. (B) An increase in caveolar ceramide promotes the association of PKB and PKC ζ and their targeting and interaction with caveolin in caveolin-enriched membrane domains. PTEN is also recruited to these membrane domains in response to ceramide. PKB is held in a repressed state within these membrane domains and is unable to support the hormonal activation of glucose transport and glycogen synthesis.

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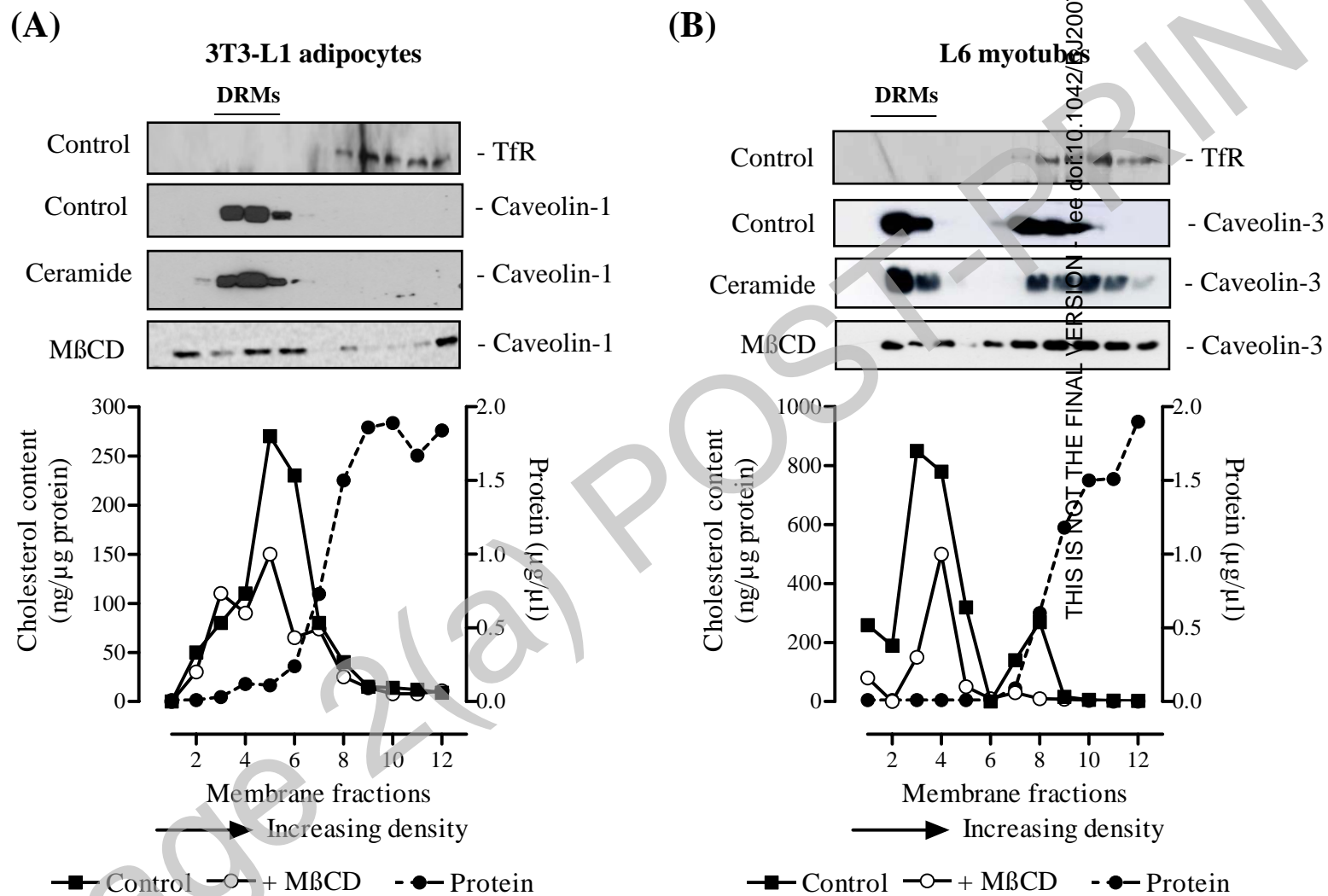
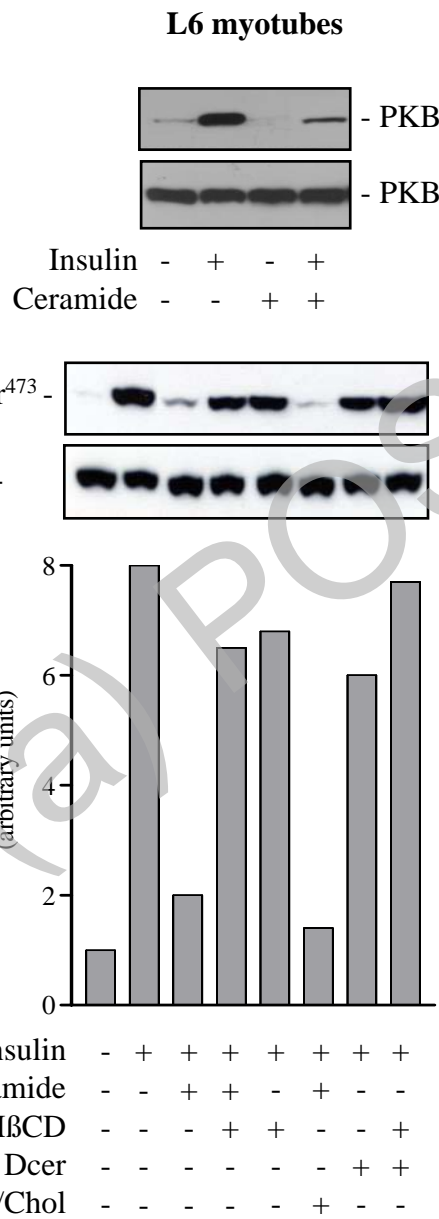
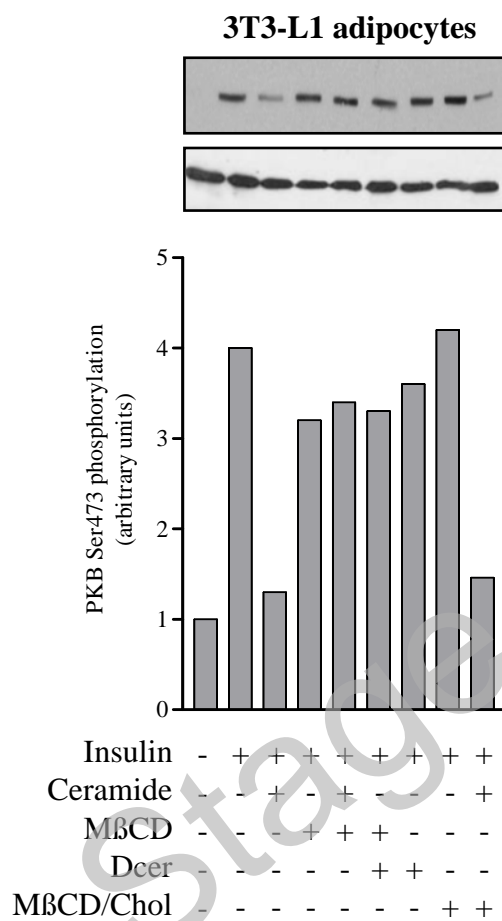
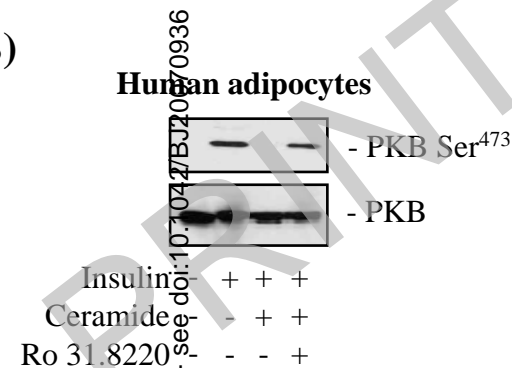


Figure 1

(A)



(B)



(C)

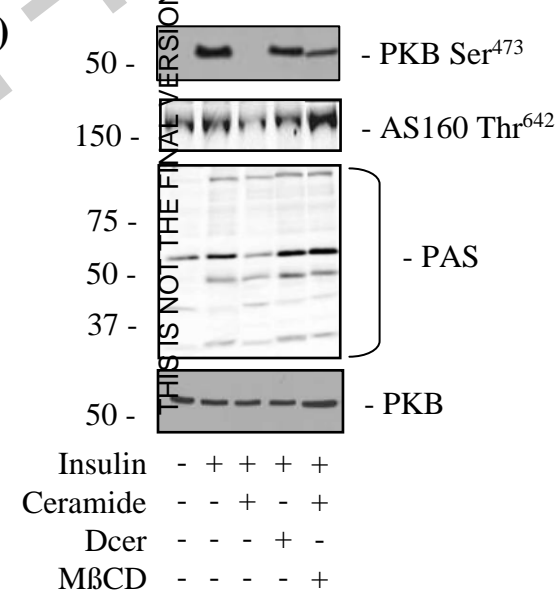


Figure 2

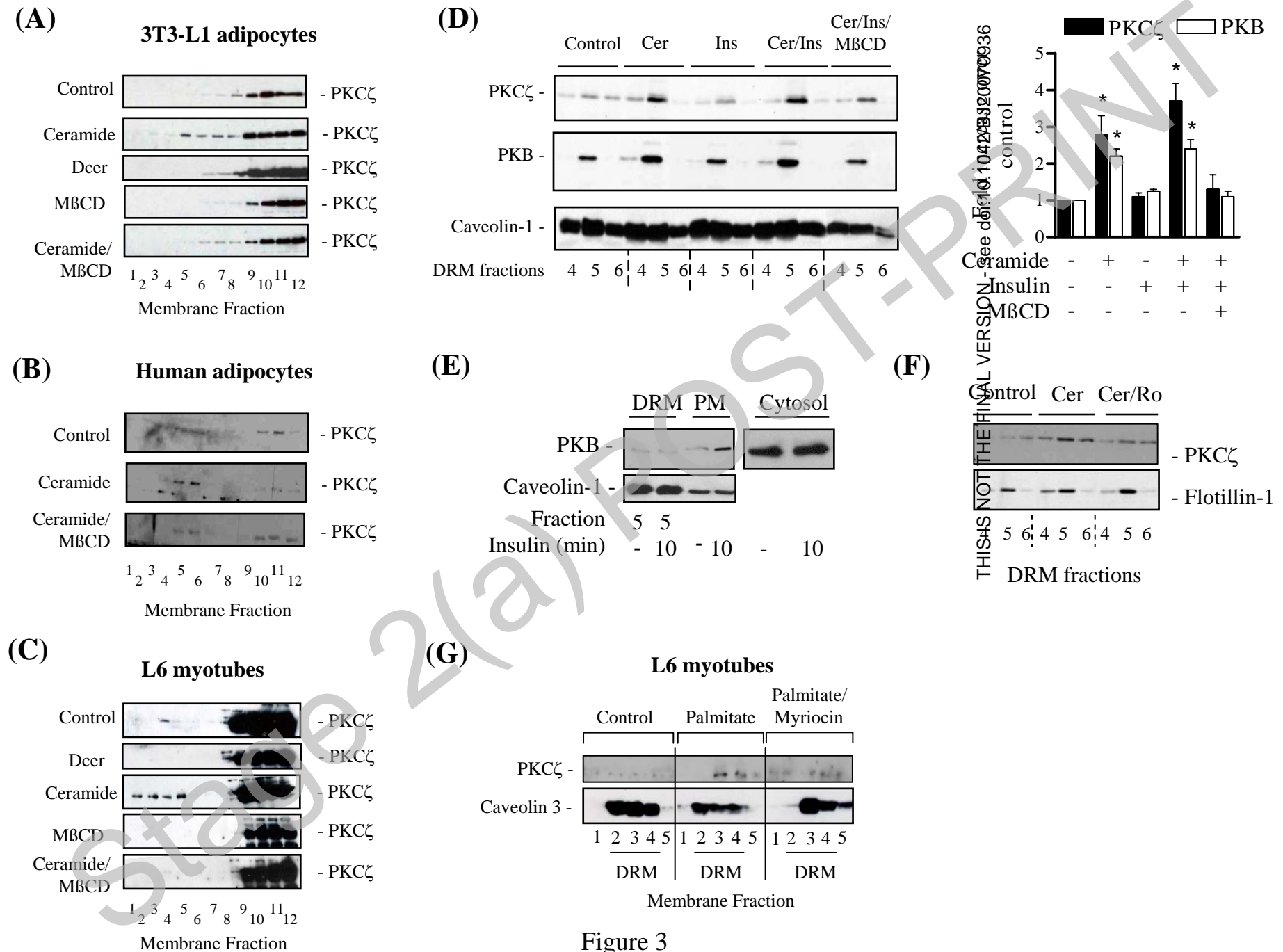
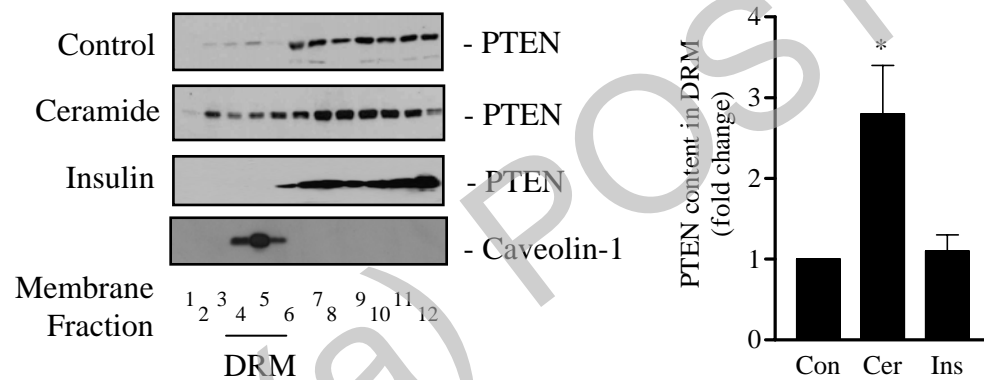


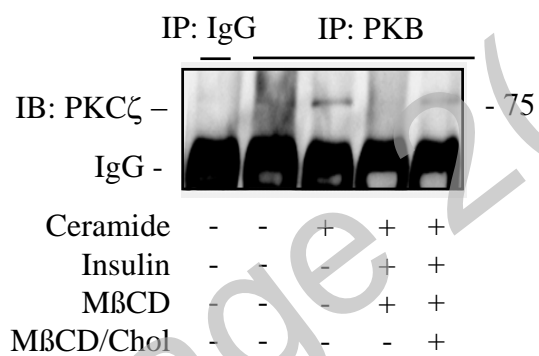
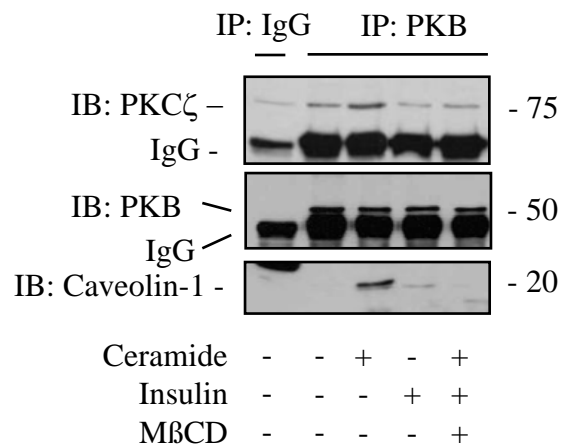
Figure 3



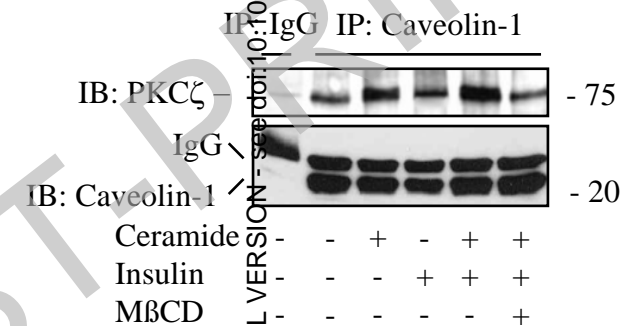
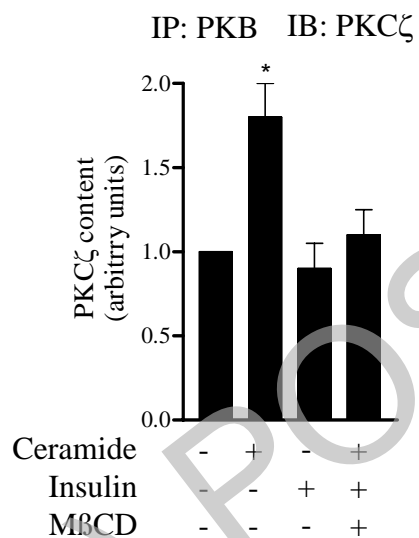
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Figure 4

(A)



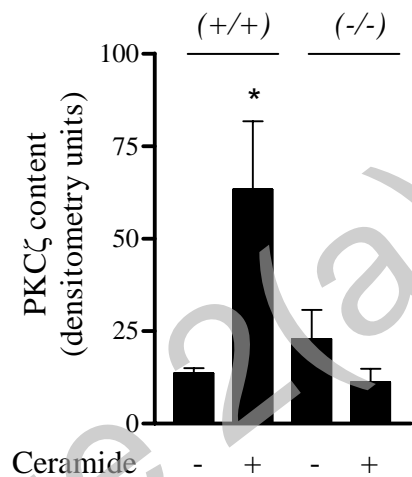
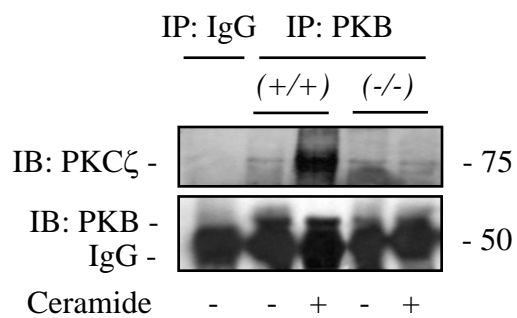
(B)



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Figure 5

(A)



(B)

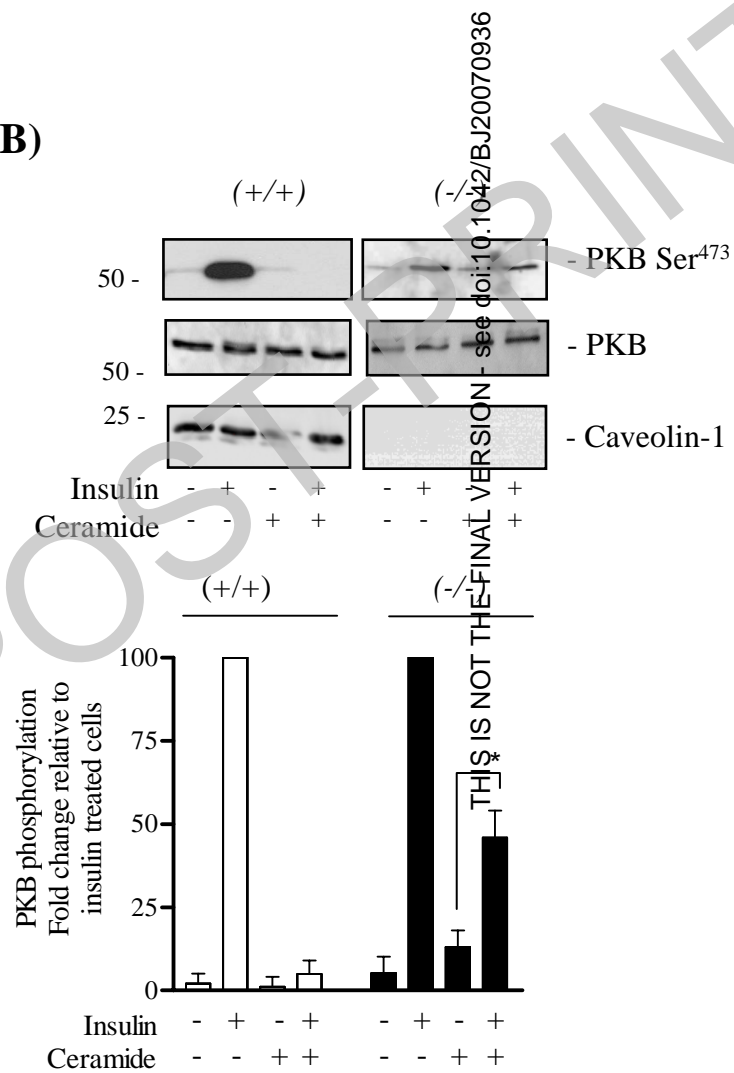
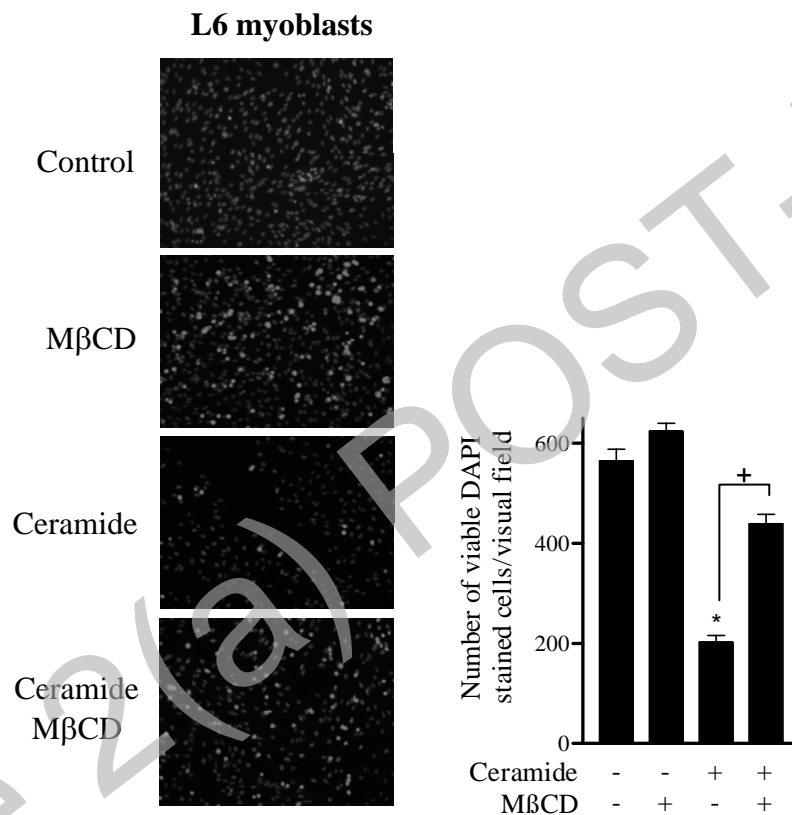


Figure 6



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Figure 7

