The metabolic consequences and sequelae of obesity promote life-threatening morbidities. PKCδ is an important elicitor of inflammation and apoptosis in adipocytes. Here we report increased PKCδ activation via release of its catalytic domain concurrent with increased expression of proinflammatory cytokines in adipocytes from obese individuals. Using a screening strategy of dual recognition of PKCδ isozymes and a caspase-3 binding site on the PKCδ hinge domain with Schrödinger software and molecular dynamics simulations, we identified NP627, an organic small-molecule inhibitor of PKCδ. Characterization of NP627 by surface plasmon resonance (SPR) revealed that PKCδ and NP627 interact with each other with high affinity and specificity, SPR kinetics revealed that NP627 disrupts caspase-3 binding to PKCδ, and in vitro kinase assays demonstrated that NP627 specifically inhibits PKCδ activity. The SPR results also indicated that NP627 affects macromolecular interactions between protein surfaces. Of note, release of the PKCδ catalytic fragment was sufficient to induce apoptosis and inflammation in adipocytes. NP627 treatment of adipocytes from obese individuals significantly inhibited PKCδ catalytic fragment release, decreased inflammation and apoptosis, and significantly improved mitochondrial metabolism. These results indicate that PKCδ is a robust candidate for targeted interventions to manage obesity-associated chronic inflammatory diseases. We propose that NP627 may also be used in other biological systems to better understand the impact of caspase-3–mediated activation of kinase activity.

The obesity epidemic continues to rise in populations worldwide. Obesity per se is not fatal; however, the metabolic consequences and sequelae of obesity promote life-threatening morbidities such as cardiovascular diseases, metabolic syndrome, type 2 diabetes, gout, and osteoarthritis. Adipose tissue is an important endocrine regulator of energy homeostasis and glucose metabolism. Fat accumulation in omental and subcutaneous abdominal depots is central to obesity and its associated diseases. Adipose tissue maintains a balance between lipogenesis (energy preservation during the postprandial period) and lipolysis (energy expenditure) via hormones and signaling pathways. Obesity results in altered profiles of adipokines (hormone and cytokines) secreted by adipose tissue. Adipose-specific adipokines include leptin and adiponectin, whereas adipocytokines such as TNFα and interleukins are secreted by adipose tissue. Adipose tissue responds to excess energy or nutrition deficiency by increasing or decreasing cell size and mass. This remodeling by adipose tissue is a dynamic process and restricts inflammation and promotes insulin sensitivity. Under conditions of excess energy leading to hypertrophy of adipose tissue, the associated macrophages acquire a pro-inflammatory phenotype. Obesity is accompanied by a chronic low level of inflammation. Apoptosis is also markedly increased in adipose tissue from obese humans and in high-fat diet–induced obesity in rodent models (1–4). This progression results in obesity-related diseases such as metabolic syndrome, diabetes, insulin resistance, and hepatic steatosis.

In obesity, the adipose stem cell (ASC) niche is altered (5). We sought to identify genes whose expression is altered in obese ASCs from obese individuals compared with lean individuals that render adipocytes susceptible to increased apoptosis and inflammation. Our previous study evaluating adipogenesis using ASCs (5) identified PKCδ as an important kinase in adipocytes. PKCδ, a member of serine/threonine kinase family, is involved in regulation of cellular differentiation, growth, and apoptosis (6, 7). PKCδ has also been shown to regulate inflammatory responses, neurotoxicity, and B cell tolerance; thus, PKCδ plays pivotal roles in normal cellular processes and in...
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diseases such as diabetes, sepsis, neurodegenerative diseases, obesity-related metabolic dysfunction, cancer, and stroke (5, 8–17). The primary amino acid structure of PKC\(\delta\) can be divided into conserved regions (C1–C4) separated by the variable regions (V1–V5). Its regulatory (N-terminal) and catalytic (C-terminal) domains are separated by a hinge region. There are several alternatively spliced variants of PKC\(\delta\) (PRKCD). We have shown that PKC\(\alpha I\) (ubiquitously expressed in all species), referred to as PKC\(\delta\) in most literature, promotes apoptosis in adipocytes (5, 18). We have shown that PKC\(\alpha II\) (mouse splice variant) and PKC\(\delta VIII\) (human splice variant) function as pro-survival proteins (19, 20); the functions of other PKC\(\delta\) splice variants are not yet been established. PKC\(\alpha I\) participates in the regulation of early stages of apoptosis by phosphorylating key apoptotic proteins or in later events by acting downstream of caspases.

PKC\(\alpha I\) has been shown to mediate inflammation in several cell types, including adipocytes, macrophages, vascular smooth muscle cells, and hepatocytes (14, 21–25). PKC\(\alpha I\) is proteolytically cleaved by caspase-3 at its hinge region, separating the regulatory domain from the catalytic domain (26–28). The release of the catalytically active fragment induces nuclear fragmentation and apoptosis (29). Cleavage and activation of PKC\(\alpha I\) are critical for its function in mediating signaling cascades in cells (30–32).

For \textit{in vivo} therapeutic applications, Rottlerin, the previously used inhibitor, has been shown not to be specific for PKC\(\alpha I\) (33). We previously published antisense oligomers (ASOs, Ionis Pharmaceuticals) directed toward the splice sites for PKC\(\delta\) that could selectively inhibit the specific splice variant (20). We showed that ASO71 decreased PKC\(\alpha I\), which resulted in a decrease in PARP cleavage (apoptosis marker). The antisense oligomer ASO71 (which decreases PKC\(\alpha I\)) and the commercially available PKC\(\alpha I\) siRNA (which decreases both PKC\(\alpha I\) and PKC\(\delta VIII\)) are not suitable, as these approaches decrease the total PKC\(\alpha I\) levels and cannot delineate the role of PKC\(\alpha I\) kinase activity specifically in obesity. Moreover, PKC\(\alpha I\) levels regulate the cell cycle and adipogenesis (6, 35); hence, it is not advisable to deplete cells of total PKC\(\alpha I\).

Our strategy was to inhibit the kinase activity of PKC\(\alpha I\), as it has been shown that PKC\(\alpha I\) activity is increased in adipocytes of obese mice (36–38). As described above, PKC\(\alpha I\) is a serine/threonine kinase that is activated by cleavage at its hinge region, releasing its C-terminal catalytic fragment. This fragment, freed from inhibition by the regulatory domain, is sufficient for its function (7). The cleavage and activation of PKC\(\alpha I\) set up a positive feedback loop that impinges upon upstream components of the death effector pathway, thereby amplifying the caspase cascade and helping cells commit to apoptosis (30–32). Hence, to specifically inhibit PKC\(\alpha I\)-mediated pathways, we chose to inhibit the kinase activity rather than decreasing its levels.

Here we identified a small molecule that would inhibit cleavage of PKC\(\alpha I\) in adipocytes and thereby decrease its kinase activity. We investigated the binding affinity of this small molecule to PKC\(\alpha I\) and its ability to inhibit binding of caspase-3 to PKC\(\alpha I\). Using this small molecule to inhibit PKC\(\alpha I\) kinase activity, we investigated whether inflammation, apoptosis, and metabolic dysfunction associated with obesity could be reduced in human adipocytes.

Results

PKC\(\alpha I\) is increased in obese human ASCs and adipocytes

We determined the expression of PKC\(\alpha I\) in human adipose tissues obtained from obese individuals. Omental and subcutaneous adipose tissue from six lean and six obese patients (IRB 20295; obese BMIs between 43 and 45 kg/m\(^2\); lean BMIs between 22 and 23 kg/m\(^2\); non-diabetic, nonsmokers, other criteria matched; designated as subcutaneous lean, subcutaneous obese, omental lean, and omental obese according to depot and lean/obese status) were evaluated for PKC\(\alpha I\) levels, and our results showed increased PKC\(\alpha I\) levels in both subcutaneous and omental depots of obese subjects (Fig. 1a). Adipose tissue was digested with collagenase and purified to obtain adipocytes (from other cells and macrophages) in supernatant, whereas the pellet contained the SVF fraction from which ASCs were isolated. The omental adipocytes were further analyzed by Western blotting. Our results showed increased PKC\(\alpha I\) expression and release of its C-terminal catalytic fragment in obese adipocytes. Obese adipocytes had increased cleavage of caspase-3 (increased apoptosis) and higher expression of the adipokine TNF\(\alpha\), indicating increased inflammation (Fig. 1b).

Next, the omental ASCs were differentiated into mature adipocytes \textit{in vitro} as described previously by our laboratory (5). We measured PKC\(\alpha I\) levels using SYBR Green qPCR, and our results showed that PKC\(\alpha I\) was increased in obese human ASCs and adipocytes (Fig. 1c).

Homology modeling and screening of small molecules binding to PKC\(\alpha I\)

Schrödinger PRIME software was used to perform homology modeling of PKC\(\alpha I\). 3D structures of PKC\(\alpha I\) isoforms (39) and crystal structures available on PDB, including 1YRK and IPTQ, were utilized to create computational chimeras to establish a suitable protein scaffold for docking. These structures were then minimized using the Schrödinger Protein Preparation Wizard and the OPLS 2005 forcefields. \textit{De novo} protein predictions were also prepared and minimized using Protein Preparation Wizard I-TASSER (40) and RAPTOR X (41).

Compounds contained in Chembridge Microformat were prepared for docking to PKC\(\alpha I\) by Schrödinger LigPrep. Schrödinger Glide XP was used to screen successful docking events based on the criteria for ligand binding to key PKC\(\alpha I\) amino acids and affinity to DXXD(P4-P1)/X (the caspase-3 recognition sequence on PKC\(\delta\) (26)). Results were tabulated using a spreadsheet, and compounds with low \(\Delta G\) across the models created were selected for \textit{in vitro} analysis. The leading hits were tested for their ability to inhibit release of the catalytic fragment of PKC\(\alpha I\) by treating obese adipocytes with 10 nm compounds. The top candidate, \(N’ \sim 1\sim, N’ \sim 5\sim-\text{bis}[3\sim-1,3\sim-dioxo\sim-1,3\sim-dihydro\sim-2\simH\sim-isindol\sim-2\sim yl][\text{propanimidoyl}]\) pentanediyhizamide (hereafter referred to as NP627), was selected (Fig. 2, \textit{a and b}) as a small molecule inhibiting the release of the PKC\(\alpha I\) C-terminal fragment. Compound 594 (Cpd594) and compound 118 (Cpd118) were from the same screening process. Cpd594 was used as a control in subsequent experiments, as it is essentially
Figure 1. PKCδι is increased in adipose tissue and adipocytes in obesity. 

(a) PKCδι levels by adipose tissue depot

(b) PKCδι cleavage in adipocyte lysates

(c) PKCδι levels in ASC and adipocytes

Figure 1. PKCδι is increased in adipose tissue and adipocytes in obesity. a, adipose tissue was obtained from lean (BMI 22–23 kg/m²) and obese (BMI 43–45 kg/m²) donors (n = 6 each group), digested with collagenase, and purified to obtain adipocytes and ASCs. Total RNA was isolated from different depots: subcutaneous lean, subcutaneous obese, omental lean, and omental obese. Real-time qPCR was performed in triplicate to measure absolute quantification of PKCδι expression using β-actin as an internal control. b, Western blotting was performed on omental adipocytes that were immunoblotted using antibodies against PKCδι, TNFα, caspase-3, and β-actin. Experiments were repeated five times with similar results. The graph represents normalized densitometric units of PKCδι_F (full-length) and PKCδι_C (cleaved) normalized to β-actin obtained in the immunoblots. c, omental ASCs were differentiated in vitro to mature adipocytes. RNA was isolated from ASCs, and adipocytes and PKCδι expression was measured using SYBR Green qPCR. The experiment was repeated five times with similar results. Statistical analysis was performed by two-way analysis of variance. ***, p < 0.001.
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Figure 2. NP627 decreased cleavage of the PKCθ C-terminal fragment. a, structure of the symmetrical PKCθ inhibitor NP627 and the control compound Cpd594, which is a monomer of NP627. b, Cpd118 and Cpd594 are from the same screening process as NP627. Western blots were performed on omental adipocytes treated with 10 nM NP627, Cpd118, or Cpd594 and immunoblotted using antibodies against PKCθ and ß-actin. The graph shows normalized densitometric units of experiments repeated five times with similar results. Statistical analysis was performed by two-way analysis of variance. **, p < 0.01.

Molecular dynamics (MD) simulation for PKCθ and NP627

The structure of PKCθ obtained from homology modeling was optimized using the QwikMD plugin for Visual Molecular Dynamics (VMD) (42). The PKCθ model was equilibrated in solvent containing 150 mM NaCl for 25 ns with the root mean square deviation (RMSD) over time, indicating a deviation from the originally produced model (Fig. 3a). This is due to compaction of the protein over the course of the MD simulation, as indicated by a lowering of dihedral angle energy (Fig. 3b), as shown in Fig. 3c at 5 ns of simulation compared with Fig. 3d, which has some secondary structure additions from the original structure and is in a more compact or well-folded state.

To probe the binding motifs of PKCθ with NP627, we docked static .pdb files taken from the MD simulation at 5-ns intervals (Fig. 3e). These docking results were based on a box centered at residues DMQD of PKCθ. The predicted ΔG for binding to this region had the highest affinity at 15 ns, with the ΔG of the highest affinity in two poses indicating a predicted K_D of 627 to PKCθ of ~1 nM. Both poses are shown with ligand interaction diagrams (Fig. 3, f and g) to show the important residues for NP627 binding to the model hinge region of PKCθ.

Binding affinity of small molecules designed for PKCθ

To characterize the interaction between the lead compound NP627 (or control Cpd594) and PKCθ, surface plasmon resonance (SPR) was used. SPR has emerged as a leading powerful technique to determine the interaction and affinity between molecules, as the ligand or the enzyme need not be labeled, thus eliminating possible changes to its molecular properties. Pure recombinant PKCθ was immobilized on a chip containing a gold film layered over glass, and the compounds were introduced in a liquid phase. The changes induced in surface plasmon resonance with binding are monitored and measured as real-time detection with high sensitivity, providing information regarding the affinity, specificity, and kinetics of biomolecular interactions. The binding affinity of pure recombinant PKCθ protein to NP627 was measured using SPR, where affinity was established via a steady state at 4 s before injection stop for NP627 or Cpd594. Binding was measured as relative response units (RU) versus time and represented in a sensorgram (Fig. 4a). This real-time measurement of association and dissociation of the binding allows calculation of association and dissociation rate constants. The kinetics for NP627 were analyzed and calculated by K_D/K_A. The K_D for NP627 was at 1.2 nM for the steady state calculation and 610 pM using the kinetics data fitting a 1:1 Langmuir binding model, with R_max set to local. Bivalent analyte fits additionally yielded a K_D of about 1.2 nM, similar to steady-state calculations, which indicates symmetry of NP627 to the PKCθ binding site. Binding of the control compound Cpd594 to PKCθ showed lower-affinity binding, with steady-state affinity calculated to be 2 ± 1.8 µM. These results (Fig. 4b) demonstrate a high binding affinity of NP627 to PKCθ. Further, they indicate the importance of symmetry and avidity for robust interaction of NP627 with the binding site of PKCθ, including the DXXD(P4–P1)/X region modeled, as indicated by cpd594 binding with less affinity to PKCθ.

NP627 disrupts binding of caspase-3 to PKCθ

The small molecule NP627 was designed to inhibit binding of caspase-3 to the hinge region of PKCθ, which results in release of the catalytic domain. Hence, the binding of PKCθ to caspase-3 was determined using SPR (as described above). The results indicate a high-affinity interaction between PKCθ and caspase-3 of 8.15 ± 69 pM (Fig. 4c). Results incorporating NP627 in the same run demonstrate nearly complete inhibition of the interaction between caspase-3 and PKCθ by addition of...
NP627. To visualize the reduction of PKCβI cleavage in the hinge region with NP627, immunochemistry was performed using an antibody specific to the target site of caspase-3 on the PKCβI hinge region (amino acids 309–318). Lean ASCs were either pretreated with 10 nM NP627 or left untreated for 24 h, followed by addition of 5 nM caspase-3 for 30 min. Cells were
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(a) [Graph showing time vs. relative response units for NP627 (nM)]

(b) [Graph showing time vs. relative response units for Cpd594 (\mu M)]

(c) [Graph showing relative response units vs. [Caspase 3], M with data points for 1nM NP627 and +Caspase 3, and 1nM NP627]

(d) [Merged DAPI + p-PKC\(\alpha\) hinge images at 20x and 4x magnification for Control ASC, ASC + casp3, and ASC +NP627 + casp3]
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washed and fixed with 4% paraformaldehyde, and images were obtained using a Nikon A1R confocal microscope. Results show decreased staining of PKCδI in caspase-3–treated cells, indicating cleavage at the PKCδI hinge region. Treatment with NP627 prevented caspase-3–mediated cleavage of PKCδI, as seen in Fig. 4d.

**NP627 inhibits the kinase activity of PKCδI**

Because we established that NP627 has robust affinity for PKCδI using SPR, we sought to evaluate its effect on PKCδI activity in cells. Obese adipocytes were treated with increasing doses (1–100 nM) of NP627 for 24 h. The expression levels of PKCδI were not affected, but NP627 inhibited cleavage and release of the PKCδI C-terminal fragment. Additionally, its splice variant PKCδVIII (lacking the caspase-3 cleavage sequence), which is present in human adipocytes, was not affected by NP627 treatment (Fig. 5a). 10 nM NP627 was sufficient to inhibit release of the catalytic fragment, and this dose was used in all experiments.

To determine the effect of NP627 on the kinase activity of PKCδI, in vitro kinase assays were performed with the recombinant proteins PKCδI and myelin basic protein (MBP, a known PKCδI substrate) in a kinase buffer containing phosphatidylserine and ATP with and without 10 nM NP627 (30-min incubation prior to the assay). The results show that NP627 treatment decreased phosphorylation of myelin basic protein (Fig. 5b).

**NP627 is specific for PKCδI**

Next we sought to evaluate whether 10 nM NP627 could affect other PKC isozymes. Using a PKC activity kit, a colorimetric assay (ENZO, run in triplicate) was performed; this incorporates recombinant PKC isoforms and tetramethylbenzidine substrate (TMB), and a color develops in proportion to PKC phosphotransferase activity. Relative kinase activity is calculated as follows: (average absorbance of PKC isozyme catalyzed as follows: (average absorbance of PKC isozyme — average absorbance of blank)/quantity of pure kinase used per assay. Our data show specificity for PKCδI; other PKC isozyme activities were not inhibited by NP627 (Fig. 5c).

**NP627 is not cytotoxic**

To determine the effect of NP627 on cellular viability and cytotoxicity in ASCs and adipocytes, we performed the WST-1 assay (Roche) according to the manufacturer’s instructions. The amount of formazan dye formed directly correlated with the number of viable cells as measured by absorbance. The WST-1 assay indicated that NP627 did not cause cellular toxicity (Fig. 5d).

**Inhibiting PKCδI with NP627 decreases inflammation in obese adipocytes**

PKCδI is an important mediator of increased levels of inflammation in obese adipocytes (23). To evaluate the effect of inhibition of release of the PKCδI catalytic fragment by NP627 on inflammation, obese adipocytes were treated with 10 nM NP627 for 24 h and used in the human inflammation array (Abcam, ab134003). Among the cytokines, TNFα, MCP-1, and IL6 were dramatically reduced upon treatment with NP627. We verified the results individually using SYBR Green real-time qPCR. The macrophage chemotactic protein 1 (MCP-1) promotes macrophage infiltration in adipocytes (43) to sustain chronic inflammation. Our results (Fig. 6a) demonstrated that the levels of MCP-1 and the pro-inflammatory adipokines TNFα and IL6 declined in NP627-treated obese adipocytes.

**NP627 decreases apoptosis in obese adipocytes**

Pre-adipocytes in obesity undergo ongoing apoptosis mediated by caspase-3 (44) and have been shown to be a key link between obesity and insulin resistance (4). To determine whether NP627 inhibited PKCδI-mediated apoptosis, lean ASCs were treated with 10 nM NP627 for 24 h, followed by caspase-3 for 30 min. Cells were washed and fixed with 4% paraformaldehyde, and immunocytochemistry was performed for PARP. Images were obtained using a Nikon A1R confocal microscope. Treatment with NP627 reduced PARP levels (Fig. 6b, indicating decreased apoptosis levels. These results indicate that release of the PKCδI catalytic fragment induced apoptosis and that this could be inhibited by NP627.

**NP627 improves cellular respiration**

Obesity is associated with decreased cellular respiration. PKCδI has been shown to improve cellular respiration in obesity (36). To determine the effect of NP627 on cellular respiration in obese adipocytes, we determined the oxygen consumption rate (OCR) using a Seahorse Bioscience XF Extracellular Flux Analyzer. ASCs from obese donors were treated with 10 nM NP627, and treatment was maintained through differentiation into adipocytes. Simultaneously, lean and obese ASCs without treatments were differentiated into adipocytes. Mature adipocytes were then analyzed for mitochondrial stress. Our results show a decreased OCR in obese adipocytes compared with lean adipocytes. Treatment of obese adipocytes with
NP627 increased the OCR. Additionally, treatment with NP627 improved ATP production, maximum respiration, and percent spare respiratory capacity of obese adipocytes (Fig. 6c). The results show that decreasing PKCβI activity by treatment with NP627 improves cellular respiration and improves mitochondrial function in obese adipocytes.

NP627 decreases PKCβI activity in obese adipose tissue

Last, adipocytes were freshly isolated from human omental adipose tissue of obese donors (IRB 20295 as described above) and were treated with 10 nM NP627 for 48 h or remained untreated. Our results showed that NP627 treatment decreased PKCβI cleavage and decreased TNFα levels (Fig. 7a).

We simultaneously used annexin/PI flow cytometry to determine the effect on ongoing apoptosis in the above adipocytes isolated from obese donors with or without NP627 treatment. Our results showed decreased apoptosis with NP627 treatment in obese patient adipocytes (Fig. 7b).

Discussion

Adipose tissue plays an important role in developing a systemic inflammatory state that significantly contributes to obe-
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(a) Inflammation levels of obese adipocytes +10nM NP627

(b) Merged DAPI + PARP

(c) Spare Respiratory Capacity %

Maximum Respiration

ATP production
sity-related morbidities such as cardiovascular risk and metabolic syndrome. PKCζ is implicated in insulin resistance, diabetes, and vascular function (15, 45, 46) related to obesity and presents itself as an important kinase in the manifestation of obesity-related morbidities. PKCζ can be activated by several mechanisms. Diacylglycerol and phorbol 12-myristate 13-acetate promote its translocation to the membrane and thereby enable substrate interaction. PKCζ is activated by cleavage at its hinge region, releasing its C-terminal catalytic fragment. This fragment, freed from inhibition by the regulatory domain, is sufficient for its catalytic function (7). PKCζ activity is also modulated by tyrosine phosphorylation via Src-related kinases (47, 48). In this study, our results showed that obese adipocytes have increased cleavage of the PKCζ catalytic domain and that release of the catalytic fragment of PKCζ promoted pro-apoptosis and pro-inflammatory states in obese adipocytes. Hence, we evaluated the effect of inhibiting release of the PKCζ kinase domain in obese adipocytes. Of interest was cleavage mediated by caspase-3 on the DXXD(P4-P1)/X site on the hinge region of PKCζ because we had shown previously that the PKCζVIII alternatively spliced variant in humans, in which the caspase-3 cleavage site is disrupted, is a prosurvival protein (20, 49).

Many different strategies have been employed to increase the specificity of small molecules to particular kinases, and here we employed a drug discovery approach taking advantage of the unique caspase-3 activation mechanism of PKCζ. We screened and identified a small molecule with dual specificity domain and that release of the catalytic fragment of PKCζ promoted pro-apoptosis and pro-inflammatory states in obese adipocytes. Hence, we evaluated the effect of inhibiting release of the PKCζ kinase domain in obese adipocytes. Of interest was cleavage mediated by caspase-3 on the DXXD(P4-P1)/X site on the hinge region of PKCζ because we had shown previously that the PKCζVIII alternatively spliced variant in humans, in which the caspase-3 cleavage site is disrupted, is a prosurvival protein (20, 49).

Figure 6. NP627 reduces inflammation and increases mitochondrial respiratory fitness. a, obese adipocytes were treated with 10 nM NP627 for 24 h. RNA was isolated, and expression levels of TNFα, MCP1, and IL6 were measured using SYBR Green qPCR using β-actin as an internal control. The experiment was performed in triplicates and repeated five times. ***, p < 0.001.

b, adipocytes isolated from human obese subjects treated with 10 nM NP627 for 24 h. Cells were gated for Annexin and PI to measure apoptosis. The experiment was performed in triplicates and repeated five times. The graph is representative of experiments repeated five times with similar results. Statistical analysis was performed by two-way analysis of variance. ***, p < 0.001.

c, human ASCs from omental lean, omental obese, and omental obese treated with 10 nM NP627 were seeded at 6000 cells/well and differentiated into mature adipocytes in a Seahorse XFp cell miniplate in triplicates, and the experiment was repeated three times. Treatment with 10 nM NP627 was maintained throughout differentiation. A mitochondrial stress test was performed according to the manufacturer’s instructions, and the OCR was measured. The measurements were normalized to cell count, and analysis was performed using Seahorse Wave software and GraphPad by two-way analysis of variance. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Figure 7. NP627 decreases PKCζ activity in human adipose tissue. a, omental adipose tissue was obtained from human obese subjects, and adipocytes were isolated and treated with or without 10 nM NP627 for 48 h. Western blot analysis was performed and immunoblotted against PKCζ, TNFα, and β-actin. Shown is a graphical representation of densitometric units in the Western blots normalized to β-actin in five experiments performed independently. b, adipocytes isolated from human obese subjects treated with 10 nM NP627 for 48 h. Western blot analysis was performed and immunoblotted against PKCζ, cleaved PKCζ, and TNFα. Shown is a graphical representation of densitometric units in the Western blots normalized to β-actin in five experiments performed independently. ***, p < 0.001.
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for PKC\(\text{I}\) and the DXXD sequence (caspase-3 recognition site) so that it distinguishes its binding specifically to PKC\(\text{I}\) and not to other PKC isoforms or the alternatively spliced variant PKC\(\text{I}\text{VIII}\) in human adipocytes. NP627 binds PKC\(\text{I}\) with high affinity (\(K_D 1.3 \text{ nm}\)), and it also shows high specificity for PKC\(\text{I}\) over other PKCs, indicating that it is a good lead compound for therapies dependent on specific caspase-3 cleavage–mediated kinase activity of PKC\(\text{I}\). The SPR data indicated that the PKC\(\text{I}\) DXXD(P4-P1)/X region is symmetrical, having an aspartate separated by two amino acids in alpha helices. MD simulations showed the interaction of NP627 with PKC\(\text{I}\) and the DMDQ region. NP627 has a dramatically higher affinity for PKC\(\text{I}\) over control the compound Cpd594 (\(K_D 2 \text{ \mu M}\)), according to SPR results. NP627 has essentially two arms of the control molecule Cpd594 attached together. The affinity loss of Cpd594 resulting from lack of an opposite symmetrical side of the molecule is over 3-fold, which could indicate importance of the linked portion of the molecule. The importance of symmetry and avidity for robust interaction of NP627 to the binding site of PKC\(\text{I}\) and experiments demonstrating inhibition of PKC\(\text{I}\) kinase activity further underscore the structure–activity relationship. The importance of symmetry for NP627 could be probed in the future by replacing the propyl group with different linkers between the two amide bonds of NP627.

Our results using SPR demonstrate nearly complete inhibition of the interaction between caspase-3 and PKC\(\text{I}\) by NP627, indicative of protein–protein inhibition, which makes NP627 one of a select few small organic molecules able to affect macromolecular interactions, such as those between protein surfaces (50). SPR data indicated binding of NP627 to PKC\(\text{I}\) at 1 \(\text{nm}\); however, our cellular assays showed efficacy at 10 \(\text{nm}\). It may be postulated that, because of the relatively high hydrophobicity of NP627, a higher dose was required under experimental conditions. A literature search did not reveal any studies utilizing the compound (referred to as NP627 here) in cell culture or animal models of disease. Future experiments are planned to test the effectiveness, pharmacokinetics/pharmacodynamics (PK/PD) and absorption, distribution, metabolism and excretion studies of NP627 in rodent models of obesity. Our results shown here demonstrate that NP627 is not toxic to cells.

The results presented here demonstrate that NP627 disrupts caspase-3–mediated activation of PKC\(\text{I}\). NP627 is highly specific for PKC\(\text{I}\) over other human PKC\(\text{I}\) splice variants and PKC isoforms. Obese adipocytes treated with NP627 showed decreased apoptosis and inflammation. Moreover, metabolic stress is reduced, demonstrating a metabolically healthier state of obese adipocytes. Using a Seahorse XFP Analyzer, we determined that the OCRs of obese adipocytes were drastically reduced compared with lean adipocytes, reflecting the compromised metabolic state in obesity. Treatment with NP627 induced a significant increase in mitochondrial OCR, especially ATP production, maximum respiration, and percent spare capacity. The results from treatment with NP627 are consistent with an improvement in ATP production because activated PKC\(\text{I}\) has been shown to move to the mitochondrial membrane and inhibit ATP synthase (51). Improved maximum respiration and percent spare capacity reflect the cell’s enhanced flexibility to compensate for metabolic challenges. Our results from the Seahorse mitochondrial stress test are supported by previous studies (52) showing that reduced PKC\(\text{I}\) activity improves the OCR and overall mitochondrial metabolism in cardiomyocytes. Other studies have shown that metabolically healthy obese subjects have lower inflammation markers (53).

Future studies will characterize whether OCR improvement with inhibition of PKC\(\text{I}\) kinase activity by NP627 is due to increased mitochondrial functionality, mitochondrial number, or other changes within the adipocyte itself. Our results presented here demonstrate that PKC\(\text{I}\) is a robust candidate for targeted intervention for management of chronic inflammatory diseases associated with obesity, such as cardiovascular diseases and diabetes. As a broader effect, the strategy of dual binding for targeting a protein kinase may result in highly specific therapy where the role of protein kinases is pivotal in disease manifestation.

Experimental procedures

Adipose tissue samples

White adipose tissue was obtained as discarded tissue from surgeries performed at Tampa General Hospital by Dr. Murr. Donors consented to their waste tissue being used in research. The subcutaneous and omental depots were collected from the same subject. Adipose tissue was obtained from lean (BMI 22–23 kg/m\(^2\)) and obese (BMI 43–45 kg/m\(^2\)) donors (n = 6 each group); both groups were nonsmokers, had no cancers, and other criteria matched. The deidentified samples were obtained under an institutional review board–approved protocol (University of South Florida IRB 20295) with a “not human research activities” determination and were transported to the laboratory and processed within 24 h of collection. Additional tissue with the same criteria was also obtained from ZenBio.

ASCs

ASCs were isolated as described previously by our laboratory (54). Briefly, adipose tissue was cut into small pieces and digested with 0.075% collagenase type I (Worthington) in modified PBS for 2 h at 37 °C. Digestion was stopped by adding \(\alpha\)-minimum Eagle’s medium and 20% heat-inactivated FBS. The suspension was filtered and centrifuged at 400 \(\times\) g at room temperature. The pellet contained the stromal vascular fraction (SVF). The pellet was resuspended in 1 ml of erythrocyte lysis buffer (Stem Cell Technologies) for 10 min and washed in 20 ml of PBS with 2% P/S/A before centrifugation (300–500 \(\times\) g, 5 min). The supernatant was aspirated, and the cell pellet was resuspended in 3 ml of stromal medium (\(\alpha\)-minimum Eagle’s medium, Mediatech) with 20% FBS, 1% l-glutamine (Mediatech), and 1% penicillin/streptomycin/amphotericin B. Following three rinses in stromal medium, SVF cells were plated for initial cell culture at 37 °C with 5% CO\(_2\) in ASC medium from ZenBio (catalog no. PM-1). Subconfluent cells were passaged by trypsination. Experiments were conducted within passages 2–3.

In vitro differentiation of ASCs to adipocytes

Adipose stem cells derived from normal or obese patients were purchased from ZenBio. These were tested in culture to...
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differentiate into mature adipocytes, show accumulation of lipid, and secrete adiponectin and leptin. At the start of all experiments, cells were grown to confluence so that all cells were synchronized and then differentiated. The cells were cultured as follows. ASCs were passaged with preadipocyte medium (PM-1; DMEM/Ham’s F-12 medium, HEPES, FBS, penicillin, streptomycin, and amphotericin B; ZenBio) and then plated at 50,000 cells/cm\(^2\) with PM-1. Cells were fed every other day with PM-1 until confluent. To induce differentiation, PM-1 was replaced with differentiation medium (DM2, ZenBio), which included biontin, pantothenic acid, human insulin, dexamethasone, isobutyryl-methylxanthine, and a PPAR\(\gamma\) agonist (days 0–7). After 7 days, DM-2 was replaced with adipocyte medium (AM1, ZenBio, days 7–14), which included PM-1, biontin, pantothenic acid, human insulin, and dexamethasone. By day 14, cells contained large lipid droplets and were considered mature adipocytes. Cells were maintained at 37 °C in a humidified 5% \(\mathrm{CO}_2\) atmosphere.

Quantitative real-time qPCR

Total RNA was isolated from cells using TRIzol (Thermo Fisher Scientific) according to the manufacturer’s instructions. 1 \(\mu\)g of RNA was used to synthesize first-strand cDNA using oligo(dT) primers and the Omniscript\textsuperscript{TM} kit (Qiagen). 1 \(\mu\)l of cDNA was amplified by real-time quantitative PCR using Maxima SYBR Green/Rox qPCR Master Mix (Thermo Scientific) in an ABI ViiA7 sequence detection system (PE Applied Biosystems) to quantify the relative levels of transcripts in the samples. The primers were as follows: PKC\(\alpha\), 5’-ACATCTTAGTAGTACAAACACGGGAC-3’ (sense) and 5’-ACCGTCTCTTCTCAGACAC-3’ (antisense); MCP1, 5’-CTCATAGCCACACACCTTCATTCC-3’ (sense) and 5’-TCAAGTCTCTCGAGTTTGGTTT-3’ (antisense); IL6, 5’-AGACAGCCACCTACACTCTTTCAG-3’ (sense) and 5’-TTCTGCCAGTGGCCTTGTTCGTC-3’ (antisense); TNF\(\alpha\), 5’-CTCTTCTCGCCCTGTGCACTTTG-3’ (sense) and 5’-ATGGGCCTACATCCGTTGCACTCT-3’ (antisense); \(\beta\)-actin and GAPDH as the endogenous control and ASC control samples as calibrator samples. Concentrations were optimized to give the desired standard curve and a single melt curve, the relative quotient was determined using the \(\Delta\Delta\text{C}_{\text{t}}\) method with \(\beta\)-actin or GAPDH as the reference gene. Absolute quantification of PKC\(\alpha\) expression levels was calculated by normalizing the values to GAPDH.

Western blot analysis

Cell lysates (40 \(\mu\)g) were separated on a 10% SDS-PAGE gel. Proteins were electrophoretically transferred to nitrocellulose membranes, blocked with Tris-buffered saline/0.1% Tween 20 containing 5% bovine serum albumin, washed, and incubated in primary antibody at 4 °C overnight. Membranes were then washed and incubated in secondary antibody (1:5000) for 1 h at room temperature, and enhanced chemiluminescence (Pierce) was used for detection. The FluorChem M\textsuperscript{TM} (Protein Simple) imaging system was used to capture digital chemiluminescence images and process Western blots. Data were analyzed using AlphaView\textsuperscript{®} software. Primary antibodies included PARP (Cell Signaling Technology, 9542), PKC\(\alpha\) (Cell Signaling Technology, 2058S), MBP and pMBP (Upstate, 05675 and 05429, respectively), caspase-3 (Santa Cruz Biotechnology, 56053), TNF\(\alpha\) (Novus, 19532), actin (Sigma, A3884), GAPDH (Santa Cruz Biotechnology, 25778), and PKC\(\alpha\)VIII raised in the Patel laboratory (20).

Drug discovery

Schrödinger’s Maestro program (version 9.3.5) was used as the graphical user interface, and compounds contained in the ChemBridge (ChemBridge Chemicals) Microformat library were prepared for virtual screening with Schrödinger’s LigPrep program as in Ref. 55. Virtual screening was performed using Schrödinger’s GLIDE software (56) on an \(\alpha\)-helical construct of the amino acid sequence DXXD(P4-P1)/X, generated using Schrödinger Prime (57). Homology modeling was performed using PDB and Schrödinger Prime to form a PKC\(\alpha\) proteinaceous region for potential DXXD(P4-P1)/X docking, and grids were generated using Glide both with and without homology-modeled PKC\(\alpha\). Schrödinger SiteMap was used on both grids to determine areas of the model suitable for binding, and docking was performed on the DXXD(P4-P1)/X region from three potential trajectories suitable for the proteinaceous DXXD(P4-P1)/X–PKC\(\alpha\) homology model. Results were then compared, and g scores were analyzed prior to ordering potential leads from ChemBridge Chemicals based on affinity for either DXXD(P4-P1)/X–PKC\(\alpha\) homology model. I-TASSER results for the PKC\(\alpha\) sequence taken from Uniprot were equilibrated for 25 ns with constant temperature with NAMD 2.12 (58) using the CHARMM36m force field (59) and as described in Ref. 60. The long-range electrostatic forces were evaluated using the particle mesh Ewald method (34). VMD 1.9.3 was used for MD RMSD trajectory visualization and analysis.

Surface plasmon resonance

SPR measurements were performed on a Biacore T200 instrument equipped with CM-5 chips. Small-molecule dissociation constants were obtained by cross-linking ~12,000 RU of PKC\(\alpha\)l to the CM5 chip using 1-ethyl-3-(3-dimethylaminopropyl carbodiimide and N-Hydroxy succinimide (EDC/NHS) chemistry. Small molecules were dissolved in running buffer of HEPES (pH 7.4) and 150 mM NaCl (HBS buffer) according to
the relative logP supplied by Chembridge. Steady-state injections were performed in HBS buffer at a flow rate of 30 μl/min with an association time of 120 s and dissociation time of 120 s, and binding was measured in relative response units as described in Ref. 60. Regeneration with 10 mM NaOH in HBS buffer was performed at flow rate of 30 μl/s for 30 s after each small-molecule injection. The steady state was obtained using GE BIAcore T200 evaluation software version 3.0 (BIAevaluate), and steady-state data were fitted and exported using BIA-Evaluate software into GraphPad Prism 7.00 for Windows (GraphPad Software, La Jolla, CA). Kinetic injections were performed in HBS buffer at 60 μl/min with an association time of 300 s and dissociation time of 120 s, followed by a 30-s injection of 10 mM NaOH in HBS buffer. Kinetics were fit using a BIAevaluate 1:1 binding model with Rmax set to local, and the resulting sensorgrams and fits were exported into GraphPad. Caspase-3 binding to PKCδ was performed by cross-linking ~800 RU of PKCδ to a CM-5 chip using EDC/NHS chemistry. Binding of caspase-3 to PKCδ and inhibition of caspase-3 binding by NP627 was performed in the presence and absence of small molecules injected at 60 μl/min with an association time of 90 s and dissociation time of 150 s as described above.

**Immunocytochemistry**

Adipocytes were plated in a 96 well-plate and treated with 10 nm NP627 for 24 h. Caspase-3 (15 units) was added for 30 min, and cells were fixed by removing medium, washing three times with PBS, and adding 4% paraformaldehyde for 30 min. Cells were blocked with 10% goat serum for 1 h and incubated overnight with primary antibodies (1:500 PARP, Upstate, 04-576; 1:500 for the pPKCδ hinge region, Santa Cruz Biotechnology, sc-377560). Cells were washed 12 times with PBS at three times and incubated for 1 h with Alexa Fluor 488 secondary antibody (1:2000, Invitrogen) at room temperature. Cells were washed with PBS and stained with DAPI. Images were captured using a Nikon A1R confocal microscope and analyzed using NIS software.

**PKC kinase activity assay using a kit from ENZO and performed according to the manufacturer’s instructions**

Briefly, the assay is based on a solid-phase ELISA that utilizes a specific synthetic peptide as a substrate for PKC and a polyclonal antibody that recognizes the phosphorylated form of the substrate. The assay is designed for analysis of PKC isozyme activity in the solution phase. In the assay, the substrate, which is readily phosphorylated by PKCs, is precoated on the wells of the provided PKC substrate microtiter plate. The samples to be assayed are added to the appropriate wells in triplicate, followed by addition of ATP to initiate the reaction. The kinase reaction is terminated, and a phosphospecific substrate antibody is added to the wells that binds specifically to the phosphorylated peptide substrate. The phosphospecific antibody is subsequently bound by a peroxidase-conjugated secondary antibody. The assay is developed with TMB, and a color develops in proportion to PKC phosphotransferase activity. Color development is stopped with acid stop solution, and the intensity of the color is measured in a microplate reader at 450 nm.

**Annexin V/PI apoptosis assay**

Adipocytes from obese were maintained in (AM) adipocyte maintenance medium (Zenbio) and treated with 10 nm NP627 for 48 h. Medium was collected, and cells were washed once with Hank’s balanced salt solution (HBSS) and then trypsinized for 5.0 min. 5 ml of complete medium was added to neutralize the trypsin. Medium and washes were pooled and centrifuged at 1200 rpm for 5 min. Cells were washed once with PBS and once with binding buffer and then incubated for 15 min with 5.0 μl of Annexin V–FITC and 5.0 μl of PI in 100 μl of binding buffer (BD Pharmingen) at room temperature in the dark. 400 μl of binding buffer was added, and cells were analyzed by flow cytometry within 1 h. Annexin V–FITC and PI fluorescence were measured using an Accuri C6 flow cytometer.

**Oxygen consumption rate**

ASCs from lean and obese donors were plated into a Seahorse XFp cell culture miniplate (Agilent Technologies) at a density of 6000 cells/well as determined by optimization cycles. The following day, NP627 was added to pre-adipocyte medium at a concentration of 10 nm and maintained with medium changes and differentiation into mature adipocytes. Adipocytes were incubated in Seahorse XF medium (supplemented with 100 mM pyruvate, 200 mM glutamine, and 2.5 mM glucose) in a non-CO2 incubator at 37°C for 1 h. Seahorse sensor cartridges were prepared and loaded into ports as described for the mitochondrial stress test (100 μM oligomycin, 100 μM carboxy cyanide p-trifluoromethoxyphenylhydrazone, and 50 μM antimycin A/rotenone). Cells were run in the Seahorse XFp Analyzer. After the Seahorse run, adipocytes were counted using a Cellometer Vision CBA Image cytometer (Nexcelom). The measurements were normalized to cell counts, and data were analyzed using Agilent Wave software.

**Statistical analysis**

All experiments were repeated three to five times to ensure reproducibility of results. Analyses were performed using PRISM™ software and analyzed using two-tailed Student’s t test or two-way analysis of variance as indicated in the figure legends; *, p < 0.05 (significant); **, p < 0.001 (highly significant); ***, p < 0.001 (extremely significant). Analysis was performed either within groups or between groups as determined by the experiment.

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