Antidiabetogenic Effects of Chromium Mitigate Hyperinsulinemia-Induced Cellular Insulin Resistance via Correction of Plasma Membrane Cholesterol Imbalance

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Abstract
Previously we found that a loss of plasma membrane (PM) phosphatidylinositol 4,5-bisphosphate (PIP$_2$)-regulated filamentous actin (F-actin) structure contributes to insulin-induced insulin resistance. Interestingly, we also demonstrated chromium picolinate (CrPic), a dietary supplement thought to improve glycemic status in insulin-resistant individuals, augments insulin-regulated glucose transport in insulin-sensitive 3T3-L1 adipocytes by lowering PM cholesterol. Here, to gain mechanistic understanding of these separate observations, we tested the prediction that CrPic would protect against insulin-induced insulin resistance by improving PM features important in cytoskeletal structure and insulin sensitivity. We found that insulin-induced insulin-resistant adipocytes display elevated PM cholesterol with a reciprocal decrease in PM PIP$_2$. This lipid imbalance and insulin resistance was corrected by the cholesterol-lowering action of CrPic. The PM lipid imbalance did not impair insulin signaling nor did CrPic amplify insulin signal transduction. In contrast, PM analyses corroborated cholesterol and PIP$_2$ interactions influencing cytoskeletal structure. As extensive in vitro study documents an essential role for cytoskeletal capacity in insulin-regulated glucose transport, we next evaluated intact skeletal muscle from obese, insulin-resistant Zucker (fa/fa) rats. As insulin resistance in these animals likely involves multiple mechanisms, findings that cholesterol-lowering restored F-actin cytoskeletal structure and insulin sensitivity to that witnessed in lean control muscle were striking. Also, experiments utilizing methyl-$\beta$-cyclodextrin to shuttle cholesterol into or out of membranes respectively recapitulated the insulin-induced insulin-resistance and protective effects of CrPic on membrane/cytoskeletal interactions and insulin sensitivity. These data predict a PM cholesterol basis for hyperinsulinemia-associated insulin resistance and importantly highlight the reversible nature of this abnormality.

Introduction
A molecular framework has emerged to explain reduced cellular insulin sensitivity in cultured adipocytes (1-4), and myotubes (5), despite normal insulin receptor signaling. In particular, novel findings from our laboratory suggest that a loss of phosphatidylinositol 4,5-bisphosphate (PIP$_2$)-regulated cortical filamentous actin (F-actin) polymerization contributes to insulin-induced insulin resistance in cultured 3T3-L1 adipocytes (2) and L6-myotubes (5). Importantly, the hyperinsulinemic model system used, which closely resembles the in vivo condition manifest in diabetes, renders the cells resistant to insulin without detectable signaling perturbations. Moreover, these cells can regain responsiveness to insulin by experimentally correcting PIP$_2$-regulated cortical F-actin structure (2, 5).

Recent reports have suggested that the actin cytoskeleton in 3T3-L1 adipocytes is intimately linked to caveolae (6, 7). Caveolae are 60-80 nm flask-shaped invaginations of the plasma membrane (PM) enriched in cholesterol and sphingomyelin (reviewed in (8)). Interestingly, it has been demonstrated that hydrolysis of sphingomyelin by sphingomyelinase activates glucose transporter GLUT4 translocation and glucose transport (9-11). We found this insulin-mimetic action of sphingomyelinase resulted from a coordinated loss of PM cholesterol (11). In direct support of PM cholesterol depletion having a beneficial effect on the glucose transport system, a dose-dependent loss of PM cholesterol and gain of PM GLUT4 is observed with increasing concentrations of methyl-$\beta$-cyclodextrin ($\beta$CD) treatment (11). We have also observed this phenomena with several other cholesterol-lowering strategies such as nystatin and filipin treatments (11) and more recently with chromium picolinate (1, 12), a dietary supplement thought to improve glycemic status in insulin-resistant individuals. However, whether inappropriate increases in PM cholesterol exist under diabetic-state conditions and contribute to the loss of cellular insulin responsiveness is not known and warrants in vitro dissection and whole animal translation.

Therefore, we explored here a new scenario in which hyperinsulinemia may induce a cholesterol-laden PM that causes PIP$_2$/F-actin dysregulation and insulin resistance. We report that the PIP$_2$/F-actin perturbations and insulin resistance induced by hyperinsulinemia arise as a result of an insulin-induced increase in PM cholesterol. These novel observations were paralleled with in vitro and ex vivo study showing that the cholesterol-laden PM can be
therapeutically targeted by several cholesterol-lowering strategies.

Results
Insulin-resistant PM. We first evaluated if PM PIP$_2$ loss induced by hyperinsulinemia was associated with any measurable changes in PM cholesterol. In line with our previous PM PIP$_2$ analyses (2, 5) a 23% loss ($p<0.03$) of immunofluorescently detectable PM PIP$_2$ (Fig. 1, bar 1) was induced by 5 nM insulin for 12 h. The same conditions induced a 16% ($p<0.05$) increase in PM cholesterol (Fig. 1, bar 4). Interestingly, the reported cholesterol-lowering action of CrPic in control cells ((1, 12); Fig. 1, bar 5) was associated with a reciprocal 34% increase ($p<0.05$) in PM PIP$_2$ (Fig 1, bar 2). Notably, this CrPic action normalized the hyperinsulinemic-induced changes in PM PIP$_2$ and cholesterol to levels that were not statistically different from baseline, basal-control levels (Fig. 1, bars 3 and 6).

CrPic action & GLUT4 mobilization. These insulin- and CrPic-induced changes in PM PIP$_2$ and cholesterol were accompanied by reciprocal changes in cellular insulin sensitivity. For example, acutely insulin-stimulated control cells showed a characteristic increase in PM GLUT4 (Figs. 2A and 2B, compare panels/bars 1 and 2) and 2-DG uptake (Fig. 2C, compare bars 1 and 2). In the presence of CrPic, control basal and acute insulin-stimulated PM sheet GLUT4 detection was increased (Figs. 2A and 2B, compare panels/bars 1-4). Not appreciated with this PM sheet assay is that the majority of the CrPic-mobilized transporters in the absence of insulin are not functionally intercalated into the PM, which we previously demonstrated by biochemical and microscopic analyses (1). In support of non-functional/non-PM intercalated transporters, CrPic-treated control cells did not show increased basal 2-DG uptake (Fig. 2C, compare bars 1 and 3). In contrast, insulin-stimulated 2-DG uptake was amplified in CrPic-treated control cells (Fig. 2C, compare bars 2 and 4), as a result of insulin-elicited fusion of the CrPic-mobilized pool of GLUT4 (1). Insulin-stimulated GLUT4 translocation and 2-DG uptake were impaired 30-40% ($p<0.05$) in cells exposed to 5 nM insulin for 12 h (Fig. 2, compare panels/bars 2 and 6). Addition of CrPic to the hyperinsulinemic medium statistically mitigated ($p<0.05$) the insulin-induced decrease in insulin-stimulated GLUT4 translocation and 2-DG uptake (Fig. 2, compare panels/bars 6 and 8). This effectively restored the level of insulin responsiveness in these cells to that witnessed under control conditions (Fig. 2, compare panels/bars 2 and 8). Based on the notion that this effect resulted from the ability of CrPic to normalize PM cholesterol content of resistant cells to that of basal control cells (Fig. 1, bar 3), but not to that of CrPic-treated control cells (Fig. 1, bar 1), we next asked if further PM cholesterol reduction would increase insulin responsiveness in these hyperinsulinemic, CrPic-treated cells to that observed in control, CrPic-treated cells. Addition of 2.5 mM βCD to the CrPic-treated hyperinsulinemic cells resulted in a 33% total decrease ($p<0.05$, data not shown) in PM cholesterol, similar to the cholesterol loss induced by CrPic in control cells. The βCD-amplified loss of PM cholesterol resulted in a level of insulin-stimulated glucose transport equivalent to that induced by CrPic-mediated cholesterol reduction in control cells. (Fig. 2C, compare bars 4 and 10). Thus, these data reveal a cholesterol-dependent, statistically significant step-wise change in cellular insulin sensitivity. Comparison of bars 6, 8, and 10 reveals that as a cholesterol-laden membrane returns to normal with CrPic, cellular insulin responsiveness significantly improves ($p<0.05$), and with further βCD-mediated PM cholesterol reduction, the insulin sensitivity is further statistically amplified ($p<0.05$). The reduction of 30-40% cholesterol by either CrPic in control cells or the combined CrPic/βCD treatment in hyperinsulinemic cells did not affect basal glucose transport.

Plasma membrane/cytoskeletal connections. As recently reviewed by Parton and Simons (8), a feature of mammalian cells are caveolae that represent specialized, morphologically distinct sphingolipid-cholesterol microdomains, which are stabilized by the caveolin protein. Numerous strategies exist to study these structures, although unfortunately problems are associated with each approach and even with their
combined use (8). Thus, our next set of studies certainly requires cautious interpretation, yet seem to show PIP₂/F-actin localized at caveolin-labeled cholesterol microdomains. For example, immunofluorescent imaging shows caveolin-1 containing circular structures on PM sheets from control and insulin-resistant cells (Fig. 3A, panels 1-4). Confocal sectioning was uninformative in determining if these domains on the PM fragments were large, deep invaginations seen in electron micrographs, termed caves (13). Panels 5-8 (Fig. 3A) show a characteristic punctuate labeling pattern of PM PIP₂. Merged images of these co-labels suggest PM PIP₂ is decorating the cholesterol-rich caveolin-labeled region in control cells (Fig. 3A, panel 9). This co-localization is more apparent in the zoomed image provided in Fig. 3B (panel 1, see boxed region). Although the limit of resolution prevents distinction of whether the PIP₂ is populating the caveolin-labeled domain or regions very close, all PM sheets prepared from insulin-resistant cells showed little, if any, PIP₂ in these regions (Figs. 3A, panel 11 and 3B, panel 3). The higher magnification images (Fig. 3B) and PIP₂/caveolin-1 signal quantification revealed a marked reduction (p<0.05) in the ratio between immunofluorescence intensities of PIP₂ and caveolin-1 (Fig. 3C, compare bars 1 and 3). Note caveolin-1 labeling intensity was not affected by the hyperinsulinemic or the CrPic treatments. Therefore, we calculated the ratio of PIP₂, as well as F-actin (below), to caveolin-1, as a semi-quantitative measure of PM PIP₂. Co-treatment of insulin-resistant cells with CrPic appeared to preserve the detection of PM PIP₂ in these caveolin-enriched regions (Figs. 3A, panel 12 and 3B, panel 4). Evident in the numerous images we captured was a clear CrPic-induced elevation of PM PIP₂ in control cells, which, via the semi-quantitative analysis, represented a statistically significant elevation in and/or near these structures by 34% (p<0.05; Fig. 3C, bar 2). This was a change consistent with the globally measured CrPic-induced increase in PM PIP₂ shown in Fig. 1. Importantly, the cells cultured under hyperinsulinemic conditions in the presence of CrPic showed a quantitatively significant restoration in the PIP₂/Cav-1 ratio to a level equivalent to that seen in the control cells (Fig. 3C, bar 4).

Similar analyses suggest a localization of F-actin at the caveolin-labeled regions in insulin-responsive cells (Fig. 4A, panels 1, 5, and 9). The insulin-induced loss of PIP₂ at these membrane regions was accompanied by a disappearance of F-actin (Fig. 4A, panels 3, 7 and 11). In contrast, insulin-resistant cells treated with CrPic retained F-actin at and/or near these cholesterol-enriched regions (Fig. 4A, panels 4, 8, 12). Qualitative (Fig. 4B) and semi-quantitative (Fig. 4C) measure of F-actin and caveolin-1 labeling revealed that actin filaments were substantially diminished in insulin-resistant cells (Fig. 4B, panel 3, and Fig. 4C, bar 3), but were completely restored by CrPic treatment (Fig. 4B, panel 4 and Fig. 4C, bar 4). Interestingly, CrPic treatment of control cells did not appear to affect F-actin localization to caveolar-containing structures (Fig. 4A, panels 2, 6, 10; Fig 4B, panel 2; and Fig. 4C, bar 2). Immunoblotting whole cell lysate with an anti-actin antibody revealed that the sustained insulin exposure did not change total cellular actin content (Fig. 4D, compare lanes 1 and 3), nor did CrPic treatment affect the total actin pool (Fig. 4D, lanes 2 and 4), lending additional support to changes in actin polymerization.

**Cholesterol-dependent action of CrPic.** It is widely appreciated that the hyperinsulinemic state contributes to the progression/worsening of insulin resistance. The findings described above suggest CrPic may offer protection against this diabetogenic consequence of compensatory hyperinsulinemia. To further understand the apparent cholesterol-PIP₂-F-actin coupling, we tested the effects of experimentally changing PM cholesterol content with βCD. As we previously characterized (11), exposure of control cells to 2.5 mM βCD resulted in a 35% decrease (p<0.05) in PM cholesterol. Also, this same treatment lowered the PM cholesterol content of insulin-resistant cells to that witnessed in control cells (data not shown), mimicking the effect of CrPic (Fig. 1). Consistent with this outcome improving cellular insulin responsiveness, maximal insulin-stimulated glucose transport was restored in insulin-induced insulin-resistant cells treated...
with 2.5 mM βCD (Fig. 5, compare bars 1-3). In accord with previous 2-DG transport assays, basal 2-DG transport was not changed (data not shown) by hyperinsulinemia (2, 5), cholesterol reduction induced by βCD (14) or CrPic (1). We have also demonstrated the utility of replenishing the depleted PM cholesterol by enriching the cell culture medium with βCD preloaded with cholesterol [βCD:Chol (1, 11)]. This βCD replenishing tactic [that we denote βCD:Chol (R)], did not affect insulin-stimulated 2-DG transport in insulin-resistant cells (Fig. 5, compare bars 2 and 4). However, we observed that the reduction in PM cholesterol induced by CrPic was prevented (data not shown) and correspondingly the protective effect of CrPic on insulin-stimulated 2-DG transport (Fig. 5, bar 5) was ineffective with βCD:Chol (R) (Fig. 5, compare bars 5 and 6).

Although these and several of our other previous findings (1, 11, 12) strongly suggest that elevation of PM cholesterol impairs cellular insulin responsiveness, this has not been directly tested. The above βCD:Chol (R) experiments utilized 1 mM βCD preloaded with cholesterol and, as we have previously reported (11), this cholesterol-loaded concentration of βCD did not increase the basal-state level of PM cholesterol in control cells. In the next set of studies we modified the βCD:Chol parameters according to Christian et al. (15) to effectively overload the basal-state level of PM cholesterol. These experiments employed the use of 5 times more βCD (5 mM) in the βCD:Chol complex [that we denote βCD:Chol (L)]. We found that control cells exposed to this βCD:Chol (L) complex conditions displayed a 75 % increase (p<0.05) in PM cholesterol (Fig. 6A, compare bars 1 and 2). In parallel, both insulin-stimulated GLUT4 translocation (Figs. 6B and 6C) and 2-DG transport (Fig. 6D) were significantly inhibited in these βCD:Chol (L)-treated cells (compare bars 2 and 3). The basal-state levels of PM GLUT4 and 2-DG transport were not affected under these conditions (data not shown).

Removal of the βCD:Chol (L) complex by washing and a recovery period of 24 h normalized PM cholesterol content (Fig. 6A, compare bars 1 and 4), insulin-stimulated GLUT4 translocation (data not shown) and 2-DG transport (Fig. 6D, compare bars 2 and 6).

These same cholesterol overloading experiments clearly diminished PM PIP2 (Fig. 7 compare panels 1 and 2) and F-actin (Fig. 7, compare panels 4 and 5) localized at and/or in close proximity to caveolin-enriched PM microdomains, in a manner similar to that seen under hyperinsulinemic conditions. Also, in an analogous fashion to CrPic, the cholesterol-lowering action of βCD treatment alone preserved PIP2 (Fig. 7, panel 3) and F-actin (Fig. 7, panel 6) in insulin-induced insulin-resistant cells that lacked both PIP2 and F-actin (see Figs. 3A and 4A, see panel 11).

**Signaling-independent action of CrPic.** Our previous studies suggested that neither the hyperinsulinemic conditions nor the effects of CrPic could be attributed to decreases or increases, respectively, in insulin signaling (1, 2, 5, 12) although some studies argue CrPic amplifies insulin signaling (16-18). Here, we do not find alterations in insulin signaling through the PI3K pathway, as reflected by unaltered insulin-stimulated phosphorylation of Akt-2 (Fig. 8A) or phosphorylation of its downstream substrate AS160 (Fig. 8B). Moreover, the insulin-stimulated phosphorylation states of Akt-2 and AS160 were unaffected by CrPic. Also not observed are any alterations in early signaling events such as phosphorylation of the insulin receptor (IR; Fig. 8C) or insulin receptor substrate-1 (IRS-1; Fig. 8D) by either chronic insulin or CrPic treatment. Note that these Akt-2, AS160, IR, and IRS-1 quantification analyses represent the ratio of immunoblot band densities detected with anti-phosphorylation antibodies (i.e., α-P(Ser)-Akt2, α-PAS, α-PY20) to that measured with specific protein antibodies (i.e., α-Akt-2, α-AS160, α-IRβ, α-IRS-1). Although recent work suggests that CrPic may augment insulin signaling via lowering total cellular protein tyrosine phosphatase 1B (PTP-1B) content in isolated skeletal muscle (16), we did not observe any hyperinsulinemic- or CrPic-induced changes in this phosphatase (Fig. 8E) in 3T3-L1 adipocytes, consistent with no augmentation of tyrosine phosphorylation and IR signaling. Finally, as supported by Fig. 4D, none of the treatment conditions (CrPic or...
chronic and acute insulin) affected total cellular actin content (Fig. 8F).

Cytoskeletal defects and cholesterol increases in insulin-resistant animals. Although we have yet to evaluate whether these changes occur in primary adipocytes collected from diabetic animal models, we have previously noted similar cytoskeletal abnormalities in isolated epitrochlearis skeletal muscle from obese 8-wk old, hyperinsulinemic, insulin-resistant Zucker fatty (fa/fa) rats (5). Thus, as a start to translating our cell-based findings to the whole animal, we extended these analyses to critically test whether cholesterol overload contributed to the skeletal muscle cytoskeletal abnormalities and insulin resistance. We first evaluated cortical F-actin structure in epitrochlearis skeletal muscle, a small flat muscle in the rat forelimb optimal for visual inspection, to ensure replication of earlier findings. Results were similar to previous observations (5) with the muscles of obese rats showing markedly lower F-actin structure (Fig. 9A). Aligned with cholesterol compromising the F-actin structure, soleus muscle membrane cholesterol from these same obese rats was 23% \((p<0.05)\) higher (Fig. 9B) than lean controls. Although the epitrochlearis skeletal muscle provides a better sample for imaging, we were able to thinly slice an imaging sample from the same soleus muscle we used to measure PM cholesterol prior to the fractionation procedure. Imaging of those samples revealed the predicted reciprocal change in filamentous actin (Fig. 9B). Insulin-stimulated glucose transport measured in contralateral soleus muscle was characteristically lower in obese than lean muscle (Fig. 9C, compare bars 2 and 4). When insulin-resistant muscles were exposed to 2.5 mM \(\beta\)CD and then treated with insulin, glucose transport in the obese group was restored to levels that were equal to those achieved by the lean group (Fig. 9C, compare bars 4 and 6). Importantly, treatment of soleus muscle obtained from the obese group with \(\beta\)CD resulted in a gain in F-actin immunofluorescent intensity (Figs. 9D and 9E). As muscle extracellular \(^{14}\)C mannitol space was not affected, this \(\beta\)CD-induced cholesterol lowering of 25-40% (11) and unpublished data) did not compromise PM integrity, results consistent with use of this low-dose \(\beta\)CD reduction of PM cholesterol in cell culture (11).

Inhibition of cholesterol synthesis. We recently observed that CrPic (1, 12) activates 5’AMP-activated protein kinase (AMPK), a protein also implicated in the beneficial effects of exercise on glucose transport (recently reviewed in (19)). AMPK is also activated by the anti-diabetogenic drug metformin (20, 21), and metformin-stimulated AMPK activity has been shown to suppress expression of sterol response element binding protein (SREBP) (21), which would presumably decrease cellular cholesterol synthesis. Here, to further test our model we assessed if metformin treatment would protect cells against insulin-induced, membrane-associated insulin resistance in a manner similar to CrPic. Treatment of 3T3-L1 adipocytes with 1 mM metformin for 16 h lowered PM cholesterol by 17% \((p<0.05)\) (Fig. 10A, bar 1), and concomitant with this cholesterol loss was a 14% increase in PIP\(_2\) detection on PM sheets (Fig. 10A, bar 4). Furthermore, the increase in PM cholesterol and reduction in PM PIP\(_2\) (Fig. 10A, bars 2 and 5) induced by chronic insulin exposure was prevented by metformin treatment (Fig. 10A, bars 3 and 6). These beneficial changes in PM cholesterol and PIP\(_2\) were paralleled by increases in insulin sensitivity. Like CrPic, metformin treatment enhanced insulin-stimulated 2-DG transport by 22% \((p<0.05)\) (Fig. 11, compare bars 2 and 4) and improved 2-DG transport by 35% \((p<0.05)\) that was impaired by chronic insulin (Fig. 11, compare bars 6 and 8). Also in line with the cholesterol-dependent action of CrPic depicted in Fig. 5, reversal of the metformin-induced cholesterol reduction by \(\beta\)CD:Chol \((R)\) prevented the beneficial effect of metformin on insulin-resistant cells (Fig. 11, compare bars 8 and 10). Importantly, these studies support work using the Zucker (fa/fa) rat that showed strikingly similar findings, with metformin restoring compromised glucose transport in primary adipocytes isolated from obese, insulin-resistant rats (22).

Discussion

This report offers several novel observations. First, accompanying the hyperinsulinemia-
induced decrease in PM PIP2 is an increase in PM cholesterol content. Second, the cholesterol-lowering ability of CrPic we first reported in control cells effectively lowered the insulin-induced elevation in PM cholesterol to cholesterol levels witnessed in control, insulin-sensitive cells. Third, this PM cholesterol normalization restored PM PIP2. Fourth, this CrPic-induced re-equilibration of excess PM cholesterol and depleted PM PIP2 reconstructed cortical F-actin structure and fully restored insulin-stimulated GLUT4 translocation and glucose transport. Fifth, metformin, presumably via its documented action on AMPK, displays a CrPic-mimicking corrective effect on diabetogenic membrane insults. Finally, testing whether these cell culture-based findings translate to the whole animal revealed strikingly similar correctable membrane/cytoskeletal abnormalities in isolated skeletal muscle, a tissue responsible for approximately 80% of postprandial glucose disposal (23) and regarded as a major peripheral site of insulin resistance in diabetes (24).

Although at this point speculative, the immunofluorescence analyses of caveolin-containing structures suggest that these PM events may be localized at caveolin-enriched PM invaginations. There are, however, complicating factors that should be considered in the interpretation of these images. It is possible that the PIP2/F-actin changes are localized in non-caveolar membrane existing between clusters of caveolae. The resolution of the immunofluorescence methodology precludes a definitive interpretation, as single 60-80 nm caveolae are not detectable, but higher-order structures resulting from their clustering are visible by light microscopy (25). Nevertheless, the imaging analyses seem to intriguingly support the observed reciprocal changes in PM cholesterol and PIP2. Notably, the F-actin labeling localized in these regions has also been documented in electron micrographs (13). Studies dissecting the precise location and/or assembly of PM cholesterol-PIP2-F-actin events are warranted, yet somewhat secondary to the unambiguous effect these PM cholesterol and PIP2/F-actin changes impart on cellular insulin sensitivity.

A caveat to the PIP2 analyses is that antibody detection does not determine whether there is an actual loss of this lipid from the PM following the 12 h insulin exposure. Careful biochemical study to distill this information is currently underway. As a loss in antibody detection of total PIP2 in the PM sheets occurs ((2, 5), and see Fig. 1), it is unlikely that hyperinsulinemia actually promotes the partitioning of PIP2 to other PM regions. We feel that a more likely event may entail recruitment of one or more PIP2 binding proteins that preclude antibody detection of the affected PIP2. This possibility would also provide a basis for the loss of PIP2-regulated F-actin polymerization. Certainly other possibilities exist, as PM PIP2 pools are under numerous forms of regulation (reviewed in (26)). For example, one alternate possibility could be that a localized PIP2-hydrolysis by phospholipase C (PLC) occurs, as PLC has been reported to localize and turn over PIP2 in caveolin-enriched membrane domains (27).

Regardless of the exact mechanism by which a cholesterol-laden PM diminishes PIP2 detection, we have shown this consequence of hyperinsulinemia impairs regulation of GLUT4 translocation and glucose transport in 3T3-L1 adipocytes (2) and L6-myotubes (5), and thus precisely defining the cholesterol, PIP2, and/or cortical F-actin basis for this defect is important. In contrast to the notion that PM PIP2 may be an important lipid mediating fusion of GLUT4 vesicles with the PM, examination of acidic phospholipid requirements for SNARE (soluble N-ethylmaleimide-sensitive fusion factor attachment receptor)-dependent membrane fusion, suggests the presence of PIP2 in acceptor membranes (i.e. PM) does not enhance SNARE-mediated fusion (28). Conversely, the addition of PIP2 to the donor vesicles (i.e. GLUT4 vesicles) was stimulatory. Interestingly, phosphatidic acid (PA) in the acceptor and donor membranes were stimulatory and inhibitory, respectively. Thus, it is possible that the hyperinsulinemic loss of PM cholesterol, PIP2, and/or cortical F-actin negatively impacts the phospholipase D (PLD)-mediated generation of PA in the PM. As PLD activity in 3T3-L1 adipocytes has been reported to promote the fusion of GLUT4 vesicles with the PM and enhance insulin action (29), tests to determine
whether hyperinsulinemia impacts PLD activity and/or the PM content of PA are of interest.

With regard to the hyperinsulinemic state increasing PM cholesterol, a growing body of evidence indicates a complex interplay between growth regulating signaling pathways and lipid metabolism (30, 31). For example, recent data show that constitutive activation of the PI3K/Akt pathway stimulates sterol regulatory element binding proteins (SREBPs), key lipogenic transcription factors directly involved in the expression of genes dedicated to the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids (30, 31). These data also lend further credence to the notion that PIP$_2$ synthesis is not decreased with chronic insulin exposure. Moreover, while data presented in Fig. 8 indicate that chronic insulin exposure does not cause a significant constitutive activation of Akt-2, the isoform primarily responsible for insulin-stimulated glucose transport (32), we did observe a significant increase in basal Akt-1 phosphorylation under hyperinsulinemic conditions (data not shown). Whether this increased basal Akt-1 phosphorylation underlies the elevated PM cholesterol content is interesting and study to dissect potential interplay between Akt signaling and cholesterol biosynthesis under these conditions is warranted.

Interestingly, recent data suggest that the anti-diabetic drug metformin enhances insulin action by increasing membrane fluidity (33, 34). As we have observed following CrPic treatment, metformin has also been reported to increase GLUT4 translocation (22, 35-39). The relative enhancing effect of metformin was higher in cells incubated in 25 mM glucose rather than in 5 mM glucose, consistent with its selective action in hyperglycemic conditions in vivo (40). We have observed that the relative enhancing effect of CrPic on GLUT4 translocation is also higher in cells incubated in 25 mM glucose rather than in 5 mM glucose (12). An increase in PM cholesterol was noted in the cells cultured in 25 mM glucose and the beneficial action of CrPic was attributed to lowering PM cholesterol content to that measured in control, 5 mM glucose, insulin-sensitive cells (12). As reported for metformin (41-44), the effects of CrPic are not attributed to increased expression of GLUT4 protein but rather its translocation and/or activation state (45-47). In line with these observations, our studies herein support a shared mechanism for the beneficial effects of metformin and CrPic on cellular insulin sensitivity, with the regulation of plasma membrane cholesterol being a central component of this mechanism.

In summary, the present study suggests that PM cholesterol influences PIP$_2$-regulated cytoskeletal structure essential for insulin-regulated GLUT4 translocation and glucose transport. While intermittent study has coupled membrane fluidity to insulin sensitivity (48-50), a mechanistic understanding has remained elusive. It is an interesting thought that PM cholesterol trimming may be a common and key anti-diabetogenic action of CrPic and metformin, as well as several other antidiabetic agents known to display AMPK-stimulating and cholesterol-lowering properties [e.g.; berberine (51, 52), cryptotanshinone (53), and fibrates (54, 55)].

Materials and Methods

**Materials**- Murine 3T3-L1 preadipocytes were purchased from American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM) was from Invitrogen. Fetal bovine serum and bovine calf serum were obtained from Hyclone Laboratories Inc. (Logan, UT). Polyclonal rabbit caveolin-1 and polyclonal goat GLUT4 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal mouse phosphatidylinositol 4,5-bisphosphate antibody was purchased from Assay Designs Inc. (Ann Arbor, MI). Anti-phospho-Akt2(Ser474) antibody was from GenScript (Piscataway, NJ). Anti-Akt and anti-phospho-Akt substrate antibodies were from Cell Signaling (Danvers, MA). Anti-AS160 antibody was from Millipore (Temecula, CA). Anti-phosphotyrosine (PY20) antibody was from BD Transduction Laboratories (Lexington, KY). Anti-protein tyrosine phosphatase-1B antibody was from Upstate (Lake Placid, NY). Anti-β-actin antibody was from Cytoskeleton (Denver, CO). Rhodamine red-X/FITC-conjugated donkey anti-rabbit or goat anti-mouse antibodies were from Jackson Immunoresearch Inc. (West Grove, PA).
Unless indicated, all other chemicals were from Sigma.

**Animals**- Specific pathogen-free obese (fa/fa) and lean (Fa/fa) female Zucker rats were obtained from Harlan Sprague-Dawley at 6 weeks of age. Upon arrival, rats were housed individually in a temperature-controlled animal room maintained on a 12:12-h light-dark cycle. The rats were fed ad libitum NIH standard chow and water. All in vivo procedures performed were based on protocols approved by the Eli Lilly Institutional Animal Care and Use Committee.

**Cell Culture and Treatments**- Preadipocytes were cultured and differentiated as described previously (11). All studies were performed on cells which were between 8 and 12 days post-differentiation. Cells were treated with serum-free DMEM with or without 10 nM CrPic (Nutrition 21, Purchase, NY) or 1 mM Metformin for 16 hours. Insulin resistance was induced by incubating cells with 5 nM insulin for 12 h, in the continual absence or presence of CrPic/Metformin. Acute insulin stimulation was achieved by spiking the cells with 100 nM insulin during the last 30 min of incubation. βCD was used to remove (2.5 mM βCD) or add (1 mM-5 mM βCD loaded with cholesterol) cholesterol as previously described (11) for 30 min prior to insulin stimulation.

**PM Analyses**- Plasma membrane sheets were prepared as previously described (1, 2, 11). Briefly, following treatments, cells were fixed by incubation for 20 min in a 2% paraformaldehyde solution containing PBS (GLUT4, F-actin analyses) or TBS (PIP2 analyses), and these membranes were used for immunofluorescence. For GLUT4 immunofluorescence, incubation with a 1:1000 dilution of GLUT4 antibody was followed by incubation with a 1:50 dilution of FITC-conjugated secondary antibody, both for 60 min at 25°C. For PIP2/caveolin-1 co-labeling, incubation for 30 min at 37°C with a 1:50 dilution of PIP2 antibody was followed by washing and identical incubation with a 1:50 dilution of caveolin-1 antibody. Appropriate rhodamine red-X-conjugated and FITC-conjugated secondary antibodies were added simultaneously for 60 min at 25°C. For caveolin-1/F-actin labeling, sheets were co-incubated with 5 μg/mL of FITC-conjugated phalloidin and a 1:50 dilution of caveolin-1 antibody for 60 min at 25°C. After washing, sheets were subsequently co-incubated with 5 μg/mL of FITC-conjugated phalloidin and a 1:50 dilution of rhodamine red-X-conjugated secondary antibody for 60 min at 25°C.

Skeletal muscle was prepared and labeled as previously described (5, 56). Briefly, after 2 weeks of acclimation, rats in the postprandial state were anesthetized with 5 mg/100 g body weight sodium pentobarbital and the epitrochlearis muscles were dissected out, blotted on gauze, quickly rinsed in saline and immersed in 4% paraformaldehyde/PBS. Both epitrochlearis muscles from five lean and 5 obese rats were used, for a total of ten muscles.

PM sheet and muscle images were obtained using the Zeiss LSM 510 NLO Confocal Microscope, and all microscope settings were identical between groups. Images were quantitated with the LI-COR infrared imaging system as previously described (57, 58). To assess GLUT4 and PIP2 content, as well as co-localization of Cav-1/PIP2 and Cav-1/F-actin, MetaMorph software was employed to digitally analyze intensities on image sections. For cholesterol analysis, membrane fractions from cultured cells and soleus skeletal muscle were prepared and assayed for protein and cholesterol content, via the Bradford Assay and the Amplex Red Cholesterol Assay, respectively, as previously described (1).

**Glucose Transport Assay**- Cells were either untreated or stimulated with 100 nM insulin for 30 min and exposed to 50 μM 2-deoxyglucose containing 0.5 μCi of 2-[3H]deoxyglucose in the absence or presence of 20 μM cytochalasin B. Glucose transport was determined as previously described (2). Rats in the postprandial state were anesthetized with 5 mg/100 g body weight sodium pentobarbital. Soleus muscles were dissected out, blotted on gauze, and transferred to 25 ml Erlenmeyer flasks containing 2 ml of Krebs-Henseleit buffer (KHB) with 0.1% bovine serum albumin (BSA), 32 mM mannitol, and 8 mM glucose. The flasks were incubated in a shaking water bath maintained at 30°C for 1 h,
and were continually gassed with 95% CO₂. Muscles were initially incubated in the presence or absence of βCD (2.5 mM) for 60 min prior to incubation under basal conditions (no additions), or stimulation with insulin (13.3 nM). The muscles were then transferred to flasks containing 2 ml of KHB with 0.1% bovine serum albumin (BSA), 40 mM mannitol, 2 mM pyruvate, and the same additions as in the previous incubation, and used for measurement of glucose transport as we have previously described (56).

Electrophoresis and Immunoblot analysis- Whole cell lysate fractions were separated by 7.5% SDS-PAGE, and the resolved fractions were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). Proteins were immunoblotted with either a phospho-specific Akt2, PAS, PY20, PTP-1B or β-actin antibody. Equal protein loading was confirmed by Ponceau staining and by immunoblot analysis for either total Akt, AS160 or β-actin. All immunoblots were analyzed via LI-COR quantification.

Preparation of Total Cell Extracts and Immunoblotting- Total cell extracts were prepared from 100-mm plates of 3T3-L1 adipocytes following the appropriate treatments. Cells from each plate were washed two times with ice-cold PBS and scraped into 1 ml of lysis buffer (25 mM Tris, pH 7.4, 50 mM NaF, 10 mM Na₃PO₄, 137 mM NaCl, 10% glycerol, and 1% Nonidet P-40) containing 2 mM phenylmethylsulfonyl fluoride, 2 mM Na₃VO₄, 5 μg/ml aprotinin, 10 μM leupeptin, and 1 μM pepstatin A, then rotated for 15 min at 4ºC. Insoluble material was separated from the soluble extract by microcentrifugation for 15 min at 4ºC. To ensure equal loading, protein concentrations were determined by the Bradford method and equivalent protein amounts of each sample were loaded onto an acrylamide gel. Samples were subjected directly to SDS-PAGE, as described below.

Statistical Analyses- Values are presented as means ±SE. The significance of differences between 2-DG uptake, immunofluorescent, and densitometry means were evaluated by analysis of variance. Where differences among groups were indicated, the Neumann-Keuls test was used for post hoc comparison between groups. Statistical comparisons of the percent change of PM cholesterol and PM PIP₂ from control were performed by a one-sample (two-tailed) t test. GraphPad Prism 4 software was used for all analyses. P < 0.05 was considered significant.

Acknowledgements
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References
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Figure Legends

Fig. 1. Insulin- and CrPic-Induced Changes in PM PIP2 and Cholesterol 3T3-L1 adipocytes were pretreated without (white bars) or with (grey bars) 5 nM insulin for 12 h in the absence or presence of chromium picolinate (CrPic), which was added to the culturing medium 4 h before the 12 h insulin pretreatment for a total time of 16 h. Plasma membrane (PM) sheets and fractions were prepared and the contents of PIP2 (left group) and cholesterol (right group) were determined, respectively. Values are means ±SEM from three to seven independent experiments. a, p<0.05 vs. basal control.

Fig. 2. Insulin-Induced Insulin Resistance is improved by CrPic Cells were pretreated as described in Fig. 1. Following the 16 h pretreatments, cells were either left untreated (A, panels 1, 5, 3 and 7; B and C, -30' Ins.) or treated (A, panels 2, 4, 6 and 8; B and C, +30’ Ins.) with 100 nM insulin for 30 min. Plasma membrane sheets were prepared and GLUT4 immunofluorescence was determined (A and B) or 2-DG transport was assessed (C). Representative GLUT4 immunofluorescent images (A), digital quantitation (B), and mean values ±SEM of 2-DG transport are shown (C) from five to eight separate experiments. All insulin-stimulated transports were significantly (p<0.05) elevated over their respective controls. a, p<0.05 vs. control - Ins.; b, p<0.05 vs. control + Ins.; c, p<0.05 vs. 12 h Ins. + Ins.; d, p<0.05 vs. 12 h Ins/CrPic + Ins.

Fig. 3. Cholesterol-Laden PM Diminishes PIP2 in and/or Near Caveolin-containing PM Plasma membrane sheets prepared from 3T3-L1 adipocytes pretreated with or without 5 nM insulin in the absence or presence of CrPic, as described in Fig. 1, were co-labeled with anti-caveolin-1 (A, panels 1-4) and anti-PIP2 (A, panels 5-8) antibodies. Images showing the merged labels are shown in panels 9-12 (A). Zoomed images of the merged labels are shown in panels 1-4 (B). Relative labeling of PIP2/Cav-1 was determined by digitally quantifying immunofluorescent label intensities in >50 individual caveolin-1-containing structures using MetaMorph software (C). All microscope and camera settings were identical between groups and shown images are representative from over 30 images collected from three to five independent experiments. a, p<0.05 vs. control (bar 1); b, p<0.05 vs. 12 h Ins.

Fig. 4. Cholesterol-Laden PM Diminishes F-Actin in and/or Near Caveolin-containing PM Plasma membrane sheets were prepared from cells pretreated as described in Fig. 3 and were co-labeled with an anti-caveolin-1 antibody (A, panels 1-4) and FITC-Phalloidin (A, panels 5-8). Images showing the merged labels are shown in panels 9-12 (A). Zoomed images of the merged labels are shown in panels 1-4 (B). Relative labeling of F-actin/Cav-1 was determined by digitally quantifying immunofluorescent label intensities as described in Fig. 3 (C). Western blot of total cellular actin in the four treatment groups (D). All microscope and camera settings were identical between groups and shown images are representative from over 30 images collected from three to five independent experiments. a, p<0.05 vs. control (bar 1); b, p<0.05 vs. 12 h Ins.

Fig. 5. CrPic Action is Cholesterol-Dependent 3T3-L1 adipocytes were pretreated without (white bars) or with (grey bars) 5 nM insulin for 12 h in the absence or presence of chromium picolinate (CrPic) as described in Fig. 1. A group of cells was exposed for 30 min to either 2.5 mM βCD (βCD, bar 6) or with a βCD:Chol complex that replenishes PM cholesterol (1 mM βCD:Chol; βCD:Chol (R), bars 3 and 4), prior to a 30 min, 100 nM insulin stimulation. 2-DG transport was measured and values are means ±SEM from three to six independent experiments. a, p<0.05 vs. 30’ insulin-stimulated control (bar 1); b, p<0.05 vs. 12 h Ins. 30’ insulin-stimulated; c, p<0.05 vs. 12 h Ins/CrPic 30’ insulin-stimulated.

Fig. 6. Addition of Exogenous Cholesterol to PM Induces Insulin Resistance in 3T3-L1 Adipocytes Cells were left untreated or treated with a βCD:Chol complex that overloads the PM with exogenous cholesterol (5 mM βCD:Chol; βCD:Chol (L)) for 2 hr and then PM cholesterol (A) PM GLUT4 (B and C), and 2-DG transport (D) were determined immediately or following a wash/24-h recovery period as
indicated. Representative GLUT4 immunofluorescent images and means ±SEM are shown from five to seven separate experiments. All insulin-stimulated values were significantly elevated over their respective controls. a, \( p < 0.05 \) vs. basal control; b, \( p < 0.05 \) vs. \( \beta \text{CD (L)} \) unwashed; c, \( p < 0.05 \) vs. control + Ins.; d, \( p < 0.05 \) vs. \( \beta \text{CD (L)} + \text{Ins. unwashed} \).

**Fig. 7.** \( \beta \text{CD-Mediated Cholesterol Shuttling Mimics Chronic Insulin and CrPic Effects.} \) Cells were left untreated (Control, panels 1 and 4), exposed to 5 mM \( \beta \text{CD:Chol for 2 hr (} \beta \text{CD:Chol (L)) , panels 2 and 5} \), or pretreated with 5 nM insulin for 12 hr and then exposed to 2.5 mM \( \beta \text{CD for 30 min (} 12 \text{ h Ins. + } \beta \text{CD, panels 3 and 6} \). Following treatments PM sheets were prepared and co-labeled for caveolin-1 and \( \text{PIP}_2 \) (panels 1-3) and caveolin-1 and F-actin (panels 4-6) as described in Figs. 3 and 4. Representative images are shown from three to five independent experiments (five to seven images collected per experiment).

**Fig. 8.** Basal and Insulin-Stimulated Insulin Receptor Signal Propagation is Unaffected by the Hyperinsulinemia and/or CrPic Treatments Cells were treated as described in Fig. 1. Following treatment, whole cell lysates were prepared and subjected to Western blot analyses to assess Akt2(Ser474) phosphorylation (A), phosho-Akt substrate phosphorylation (B), insulin receptor phosphorylation (C), insulin receptor substrate-1 phosphorylation (D), and total cellular PTP-1B (E). White bars and grey bars represent control and hyperinsulinemic groups, respectively. Immunoblots shown in A and B are representative of three independent experiments and all quantitated values presented as means ±SEM from three independent experiments were determined by densitometry and normalized to either total protein (Akt2, AS160, IR, IRS-1) or total cellular \( \beta \)-actin (PTP-1B). All insulin-stimulated values were significantly \( (p < 0.05) \) elevated over their respective controls.

**Fig. 9.** Cholesterol Lowering Normalizes Insulin Sensitivity in Obese Zucker Skeletal Muscle Rat epitrochlearis muscle (A) and soleus muscle (B, C, D and E) from lean/obese Zucker rats were labeled with antibodies against F-actin, imaged by confocal microscopy (A and D), and digitally quantitated using MetaMorph software (B and E). Remaining soleus muscle was fractionated for PM cholesterol analyses (B). Contralateral soleus muscles were subjected to basal and insulin-stimulated 2-DG uptake measurements (C) as described in Material & Methods. A subgroup of these muscles were exposed to 2.5 mM \( \beta \text{CD for 30 min prior to the 30 min insulin stimulation Values are means ±SEM from five independent experiments. a, } p < 0.05 \) vs. Lean; b, \( p < 0.05 \) vs. Lean + Ins; c, \( p < 0.05 \) vs. Obese + Ins; d, \( p < 0.05 \) vs. Obese –\( \beta \text{CD} \).

**Fig. 10.** Metformin-Induced Changes in PM Cholesterol and \( \text{PIP}_2 \) 3T3-L1 adipocytes were pretreated without (white bars) or with (grey bars) 5 nM insulin for 12 h in the absence or presence of metformin (Met), which was added to the culturing medium 4 h before the 12 h insulin pretreatment for a total time of 16 h. Plasma membrane (PM) fractions and sheets were prepared and the contents of cholesterol (left group) and \( \text{PIP}_2 \) (right group) were determined, respectively. Values are means ±SEM from three to four independent experiments. a, \( p < 0.05 \) vs. basal control.

**Fig. 11.** Metformin-Stimulated Cholesterol Reduction Improves Insulin-Induced Insulin Resistance Cells were pretreated as described in Fig. 10. Following the 16 h pretreatments, cells were either left untreated (+30 min Ins.) or treated (+30 min Ins.) with 100 nM insulin for 30 min. 2-DG transport was assessed. Mean values ±SEM of 2-DG transport are shown from four to nine separate experiments. a, \( p < 0.05 \) vs. control + Ins; b, \( p < 0.05 \) vs. 12 h Ins. + Ins; c, \( p < 0.05 \) vs. 12 h Ins/Met + Ins.

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