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Rottlerin inhibits multiple steps involved in insulin-induced glucose uptake in 3T3-L1 adipocytes

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Abstract

Recently, it was shown that rottlerin inhibits insulin-stimulated glucose uptake and reduces intracellular adenosine triphosphate (ATP) levels in 3T3-L1 adipocytes, suggesting that these two events are causally linked. However, several other reports show that ATP-depletion induces glucose uptake in both muscle cells and adipocytes. In the present study, the mechanism of inhibition by rottlerin was studied in detail, in order to resolve this apparent discrepancy. It was found that rottlerin strongly reduces insulin-stimulated 2-deoxyglucose (2-DOG) uptake in 3T3-L1 adipocytes by a partial inhibition of the translocation of the insulin-responsive GLUT4 glucose transporter towards the plasma membrane (PM). Whereas the insulin-induced phosphatidyl-inositol-3′ (PI-3′) kinase signaling pathway is unaffected by rottlerin, Cbl tyrosine phosphorylation, which provides an essential, PI-3′ kinase-independent signal towards GLUT4 translocation, is markedly attenuated. Furthermore, we also observed a direct inhibitory effect of rottlerin on insulin-induced glucose uptake in 3T3-L1 adipocytes. The direct inhibition of insulin-stimulated 2-DOG uptake by rottlerin displayed characteristics of uncompetitive inhibition: with the $K_{m(app)}$ of glucose uptake reduced from 1.6 to 0.9 mM and the $V_{max(app)}$ reduced from 5.2 to 1.0 nmol/min mg in the presence of rottlerin. In conclusion, rottlerin inhibits multiple steps involved in insulin-stimulated 2-DOG uptake in 3T3-L1 adipocytes. The observed reduction in GLUT4 translocation towards the PM and the uncompetitive inhibition of the glucose transport process provide alternative explanations for the inhibitory effects of rottlerin aside from the effects of rottlerin on intracellular levels of ATP.

Keywords: PI-3′ kinase; Cbl; ATP; GLUT4; GLUT1; Fibroblasts

1. Introduction

A major response of adipocytes and muscle cells to insulin is the induction of glucose uptake by stimulating the translocation of the insulin-responsive GLUT4 glucose transporter towards the plasma membrane (PM). Upon binding to its cognate receptor, insulin induces activation of the insulin receptor (IR) tyrosine kinase domain and phosphorylation of insulin receptor substrate (IRS)-1 and -2 on multiple tyrosine residues. These tyrosine residues bind and activate phosphatidyl-inositol-3′ (PI-3′) kinase. PI-3′ kinase subsequently generates $PI(3,4,5)_3P$ which acts as an intracellular second messenger by inducing the activation of the protein kinase A, G and C superfamily (AGC) family members protein kinase B (PKB) and protein kinase C (PKC)-λ/ζ. Of note, 3T3-L1 adipocytes only express the atypical PKC-λ isoform [1,2]. Active PKB and PKC-λ subsequently provide an essential signal towards GLUT4 translocation via an as of yet unidentified mechanism.

A PI-3′ kinase-independent signal essential for GLUT4 translocation is provided by the IR-induced tyrosine phosphorylation of Cbl (reviewed in Refs. [3–5]). Insulin-induced Cbl tyrosine phosphorylation results in the activation of the small G-protein TC10 which signals towards the exocyst complex involved in GLUT4 vesicle docking and membrane fusion [6,7]. Interference with any step in this complex pathway through either pharmacological inhibition

Abbreviations: ATP, adenosine triphosphate; 2-DOG, 2-deoxyglucose; PI, phosphatidyl-inositol; AGC, protein kinase A, G and C superfamily; LDM, low density microsomal vesicles; PM, plasma membrane; BLU, Boehringer Light Unit (arbitrary); IC_{50}, inhibitory concentration 50%; IR, insulin receptor; IRS, IR substrate

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or by an affliction, such as type II diabetes, will result in a reduction of GLUT4 translocation in response to insulin, and consequently in a marked reduction of glucose uptake.

The inhibitor rottlerin was originally described as a specific inhibitor of PKC-δ and calmodulin kinase III [8]. However, this specificity has been questioned by Davies et al. who failed to observe a direct effect on in vitro PKC-δ kinase activities [9]. Furthermore, Soltoff demonstrated that rottlerin acts as a mitochondrial uncoupler [10]. Recently, Kayali et al. reported that rottlerin potently inhibited insulin-induced glucose uptake [11]. Furthermore, they also observed a reduction of intracellular adenosine triphosphate (ATP) in 3T3-L1 adipocytes after treatment with rottlerin. They suggested that the ATP-depletion could interfere with GLUT4 translocation by depriving the GLUT4 translocation machinery from ATP. Remarkably, however, other mitochondrial uncouplers and/or ATP-depletion have been shown to induce, rather than inhibit glucose uptake in both muscle cells and adipocytes [12–14]. In this manuscript, we describe the results of our studies on the effects of rottlerin on insulin-induced signaling pathways in 3T3-L1 adipocytes.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium was purchased from Life Technologies; fetal calf serum was from Brunschwig (cat. no. A15-043, lot no. A01127-318); dexamethasone, 1-methyl-3-isobutylxanthine (IBMX), bovine insulin and 2-deoxy-D-glucose were obtained from Sigma-Aldrich. [2-*deoxy*-D-^{14}C]Glucose was purchased from NEN-Dupont; rottlerin was from Calbiochem.

The phospho-specific antibodies recognizing acetyl-CoA carboxylase (ACC) (S79), AMP-activated protein kinase (AMPK) (T172), PKB (S473), PKC-λ (T403) and ERK-1/2 (T202/Y204) were obtained from Cell Signaling as was the antibody recognizing AMPK; rabbit polyclonal anti-IR, mouse monoclonal anti-phosphotyrosine, goat polyclonal anti-GLUT4 (C-20) and anti-p110α and HRP-conjugated donkey anti-goat secondary antibody were obtained from Santa Cruz; mouse monoclonal anti-Cbl (7G10) was obtained from Upstate; rabbit polyclonal antibodies recognizing IRS-1 and -2 have been described previously [15]; all HRP-conjugated secondary antibodies were from Promega.

2.2. Cell culture

3T3-L1 fibroblasts were obtained from ATCC and differentiated to adipocytes as previously described [16]. Mature adipocytes were routinely used 7 days after completion of the differentiation process. Only cultures in which >95% of cells displayed adipocyte morphology were used.

2.3. Analysis of intracellular levels of ATP

3T3-L1 adipocytes were treated as indicated in the figure legend, subsequently cells were lysed in 1% TCA. Cell lysates were tumbled for half an hour at 4 °C, cell lysate was cleared from cellular debris by spinning at 14,000 × *g*, for 10 min at 4 °C in a table-top centrifuge. Subsequently, the pH was neutralized by adding a 10th of the volume NaOH [600 mM] and buffered using 1/50th of the volume Tris–acetate [1 M], pH 7.75. The ATP concentration was determined using an Enliten ATP assay system (Promega) on a Lumat LB 9507 (Perkin Elmer) which had been calibrated against a concentration range of ATP.

2.4. Assay of 2-DOG uptake

3T3-L1 adipocytes, grown in 12-well plates (Costar), were subjected to an assay of [2-*deoxy*-D-^{14}C]glucose (0.075 μCi per well) uptake as described previously [17].

2.5. Membrane isolation assay and calculations

After treatment, cells were washed twice with phosphate-buffered saline on ice and scraped in HES-buffer (20 mM HEPES, pH 7.4, 1 mM EDTA and 250 mM sucrose) in the presence of protease inhibitors. Samples were homogenized by 9 × 3 strokes in a potter homogenizer after which low density microsomal vesicle (LDM) and PM were isolated by differential centrifugation as described by Simpson et al. [18]. Equal amounts of protein as determined using BCA protein assay reagent (Pierce) were subjected to immunoblot analysis and quantified using LumiAnalyst software on a LumniImager (Boehringer-Mannheim). Subsequently, data were corrected for protein content, and expressed as a relative fraction of GLUT4 residing in either the intracellular insulin-responsive LDM fraction or the PM fraction. Thus, graph-data are: LDM [BLU/mg]/(LDM [BLU/mg] + PM [BLU/mg]) and similar for PM, BLU is "Boehringer Light Unit", an arbitrary unit provided by the LumniImager. It is important to note that the amount of GLUT4 did not alter in any of the other fractions (such as HDM) obtained.

2.6. Immunoprecipitations and Western blotting

9-cm dishes of 3T3-L1 adipocytes were stimulated with agonists and scraped in lysis buffer (1 mM Na_3VO_4, 1 mM EGTA, 1 mM EDTA, 50 mM Tris–Cl, pH 7.4, 1% NP-40, 0.5% NaDOC, 150 mM NaCl, 5 mM NaF in the presence of protease inhibitors). Cell lysates were tumbled for half an hour at 4 °C, cell lysate was cleared from cellular debris by spinning at 14,000 × *g*, for 10 min at 4 °C in a table-top centrifuge. 1 mg of cell lysate was subjected to immunoprecipitation using 5 μg of the appropriate antibody for 1.5 h at 4 °C. Immuno-complexes were harvested by incubating with Prot-beads (Using ProtA for the rabbit

polyclonal antibodies and ProtG for the mouse monoclonal anti-Cbl) for 1.5 h at 4 °C. Beads were washed in lysis buffer and subsequently dissolved in sample buffer.

2.7. Statistical analysis and graph generation

Statistical analysis of the data obtained was performed with an independent-samples *t*-test using SPSS 10.0. Graphs were generated using PRISM 2.01.

3. Results

3.1. The effects of rottlerin on insulin-induced glucose uptake

In order to analyze the effects of rottlerin on insulin-induced glucose uptake, 3T3-L1 adipocytes were pretreated for 15 min with rottlerin. Subsequently, insulin-induced 2-DOG uptake was determined (see Fig. 1). Basal glucose uptake, which is largely mediated by GLUT1, was not significantly affected, whereas glucose uptake induced by 100 nM insulin stimulation was sensitive to inhibition by rottlerin. An IC_{50} value of 10 μM was found for inhibition of insulin-induced glucose uptake, which is fully consistent with the observations made by Kayali et al. [11]. Intriguingly, an identical IC_{50} value was observed for the effects of rottlerin on arsenite-induced glucose uptake (Fig. 1). This compound induces GLUT4-mediated glucose uptake independent of PI-3′ kinase activity [19].

3.2. The effects of rottlerin on intracellular ATP levels and related signaling pathways

Fig. 2A shows that rottlerin treatment of 3T3-L1 adipocytes caused an 80% reduction in intracellular levels of ATP, as has also been observed by Kayali et al. [11].

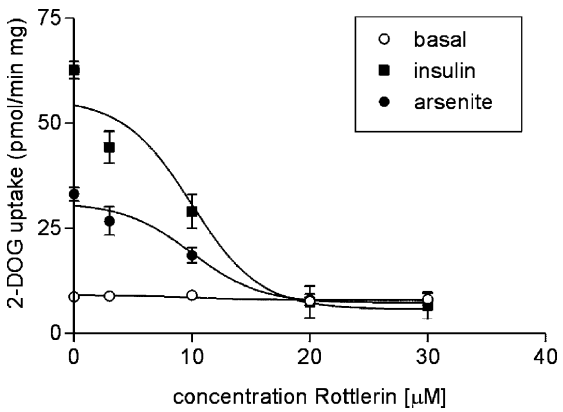

Fig. 1. Rottlerin inhibits insulin-induced glucose uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes were pretreated with the indicated concentrations of rottlerin for 15 min, subjected to stimulation with 100 nM insulin for 15 min or 0.5 mM arsenite for 30 min and assayed for [2-*deoxy*-D-^{14}C]glucose (2-DOG) uptake with 30 μM 2-DOG. Values are mean ± S.E. of three determinations performed in duplicate.

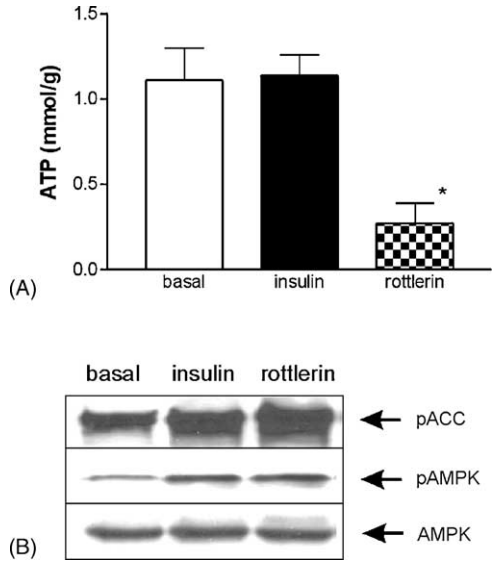

Fig. 2. The effects of rottlerin on intracellular ATP and related signaling pathways. (Panel A) 3T3-L1 adipocytes were treated with 20 μM rottlerin or 100 nM insulin for 15 min as indicated. Subsequently, adipocytes were lysed and the intracellular concentration of ATP was determined as described in Section 2. Values are mean ± S.E. of five independent observations. (Panel B) 3T3-L1 adipocytes were treated with 20 μM rottlerin for 15 min or with 100 nM insulin as indicated. Subsequently, cells were lysed and subjected to immunoblot analysis on whole cell lysate. An equal amount of protein (∼10 μg) was loaded in each lane. Blots were probed with specific antibodies recognizing phospho-S79 acetyl-CoA-carboxylase (pACC), phospho-T172 AMPKα (pAMPK) and AMPKα (AMPK) to ensure equal loading. Data shown are representative for an experiment performed in triplicate.

Insulin stimulation did not affect cellular ATP levels, as expected. In muscle cells, insulin stimulation as well as ATP-depletion are linked to the activation of AMPK and phosphorylation of its downstream target ACC [20]. As can be observed in Fig. 2B, in 3T3-L1 adipocytes a similar response occurs after insulin stimulation or rottlerin treatment.

3.3. The effects of rottlerin on insulin-induced GLUT4 translocation

Insulin increases glucose uptake in 3T3-L1 adipocytes primarily by stimulating GLUT4 translocation towards the PM. Thus, we analyzed the effects of rottlerin on translocation in more detail. Adipocytes were pretreated with rottlerin and stimulated with insulin. Subsequently, adipocytes were subjected to subcellular fractionation and the GLUT4 content of PM and intracellular microsomal vesicles (LDM) was determined by immunoblot analysis. As can be observed in Fig. 3A, rottlerin treatment did not affect basal levels of GLUT4 in the PM significantly, but attentuated (though not inhibited completely) insulin-induced GLUT4 translocation. We also analyzed the effect of rottlerin on GLUT1, as can be observed in Fig. 3C, rottlerin treatment did not attenuate GLUT1 localization. When quantifying these data, and analyzing translocation

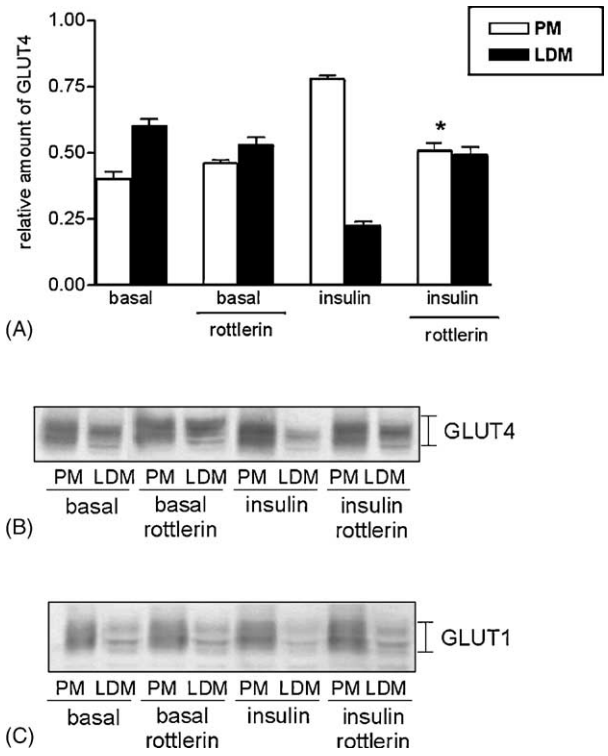

Fig. 3. Rottlerin partially inhibits insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. (Panel A) 3T3-L1 adipocytes were pretreated with 20 μM rottlerin for 15 min prior to insulin stimulation, stimulated with 100 nM insulin for 15 min and subjected to subcellular fractionation as described in Section 2. Plasma membrane (PM) and low density microsomal vesicle (LDM) fractions were harvested and subjected to immunoblot analysis with equal amounts of protein loaded for the separate fractions. Blots were probed with a specific antibody recognizing GLUT4 and quantified using a LumniImager. The respective amounts of BLU measured were expressed as relative amounts of GLUT4 residing in either PM or LDM depicted on the blot (the amount of GLUT4 residing in the HDM fraction did not alter significantly between the different stimuli). Data shown are mean ± S.E. of an experiment performed in triplicate. An asterisk (*) indicates $P < 0.05$ compared to both insulin, as well as basal. (Panel B) A representative immunoblot used to obtain the data depicted in panel A. (Panel C) A representative immunoblot probed with an antibody recognizing GLUT1.

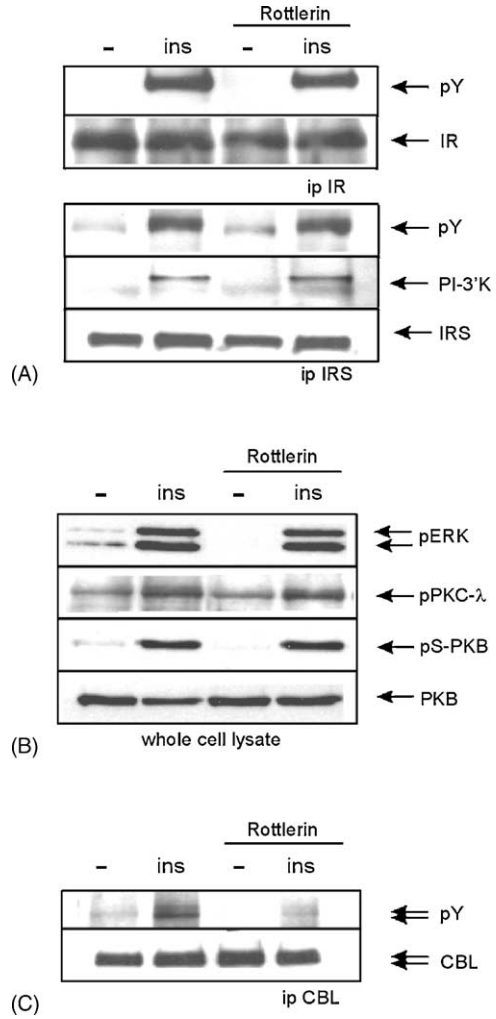

Fig. 4. The effect of rottlerin on insulin-induced phosphorylation of signaling intermediates. (Panel A) 3T3-L1 adipocytes were pretreated with 20 μM rottlerin for 15 min prior to stimulation with 100 nM insulin for 15 min and lysed as described in Section 2. Equal amounts of protein in cellular lysate were subjected to immunoprecipitation using antibodies recognizing the insulin receptor (top) or insulin receptor substrate (IRS)-1/-2 (bottom). Immunoprecipitates were subjected to immunoblot analysis using antibodies recognizing phosphotyrosine (pY), the insulin receptor (IR) or IRS-1/2 and an antibody recognizing the p110α catalytic subunit of PI-3′ kinase. Data shown are representative immunoblots of an experiment performed in duplicate. (Panel B) Whole cell lysate (~10 μg) of adipocytes stimulated as described in panel A were subjected to immunoblot analysis using antibodies recognizing phospho-T202/Y204 p44/42 MAP kinase (pERK), phospho-T403 PKC-λ (pPKC-λ), phospho-S473 PKB (pS-PKB) and PKB (PKB). Data shown are representative for an experiment performed in duplicate. (Panel C) Equal amounts of protein in cellular lysate treated as described in panel A were subjected to immunoprecipitation using an antibody specifically recognizing c-Cbl. Immunoprecipitates were subjected to immunoblot analysis using an antibodies recognizing phosphotyrosine (pY) and Cbl (CBL). Data shown are representative for an experiment performed in triplicate.

over several experiments, the incomplete inhibition of GLUT4 translocation is observed to be statistically significant ($P < 0.05$) (see Fig. 3B). When assessed in a slightly different manner, i.e. applying: $(PM_{rottlerin} - PM_{basal})/(PM_{insulin} - PM_{basal}) \times 100\%$, a partial inhibition of insulin-induced GLUT4 translocation of 74% is calculated.

3.4. The effect of rottlerin on insulin-induced signaling pathways

Insulin-induced GLUT4 translocation is dependent on two different signaling pathways. One involves Cbl tyrosine phosphorylation and the other proceeding through PI-3′ kinase activation. Therefore, we analyzed the effects of rottlerin on the insulin-induced activation of these pathways. As can be seen in Fig. 4A, the insulin-induced tyrosine phosphorylation of both IR and IRS was unaffected by rottlerin. PI-3′ kinase binding to the tyrosine-phosphorylated IRS was also still intact after rottlerin treatment (see Fig. 4A). Further downstream, also the phosphorylation of the AGC kinases PKB and PKC-λ was unaffected by rottlerin treatment (see Fig. 4B) suggesting that the PI-3′

kinase axis of insulin signaling was not influenced. On the other hand, Fig. 4C shows that the insulin-induced phosphorylation of Cbl was markedly attenuated after rottlerin treatment.

3.5. A kinetic analysis of the effects of rottlerin on glucose uptake

The data presented in Figs. 1 and 3 show that rottlerin inhibits glucose uptake by more than 90%, whereas it partially inhibits GLUT4 translocation. Therefore, we tested if rottlerin also had a direct inhibitory effect on glucose uptake. Upon insulin stimulation, GLUT4 translocates towards the PM with a $t_{1/2}$ of ~2.5 min [21]. Thus, after 15 min of stimulation with insulin the GLUT4 translocation is complete. If in a glucose uptake assay insulin is allowed to act uninhibited for 15 min followed by the addition of rottlerin concomitantly with the addition of the radiolabeled glucose, rottlerin will not exert effects through GLUT4 translocation. Still, under these conditions it was observed that rottlerin significantly reduced insulin-stimulated glucose uptake by 30%. In order to further elucidate this effect, a concentration range of rottlerin was applied (see Fig. 5A). It was observed that (under 30 μM 2-DOG, which is routinely applied in glucose uptake assays) rottlerin has a direct inhibitory effect on glucose uptake with an IC_{50} of 50 μM. (Contrasting to the fivefold lower IC_{50} when rottlerin treatment is applied prior to insulin stimulation, see Fig. 1.)

To gain further information on this direct effect of rottlerin, we performed a kinetic analysis, varying the concentration of substrate (2-DOG) under a very short incubation time with radiolabeled glucose concomitant with rottlerin (1 min). The data obtained (Fig. 5B) are a hallmark for uncompetitive inhibition, i.e. both the K_m and the V_{max} are reduced. Hence, the higher the amount of substrate added, the lower the IC_{50} value of rottlerin becomes. In the presence of rottlerin the $K_{m(app)}$ of glucose uptake is reduced from 1.63 to 0.86 mM and the $V_{max(app)}$ is reduced from 5185 to 1027 pmol/min mg. It must be observed that in these experiments, 10 μM rottlerin was added simultaneous with the label after 15 min of prestimulation with insulin. The reason for deviating from the rottlerin concentration applied throughout the manuscript can readily be appreciated from Fig. 5B. The uncompetitive effects of rottlerin being so pronounced that even 10 μM rottlerin inhibits glucose uptake for 80% in the presence of 3 mM 2-DOG.

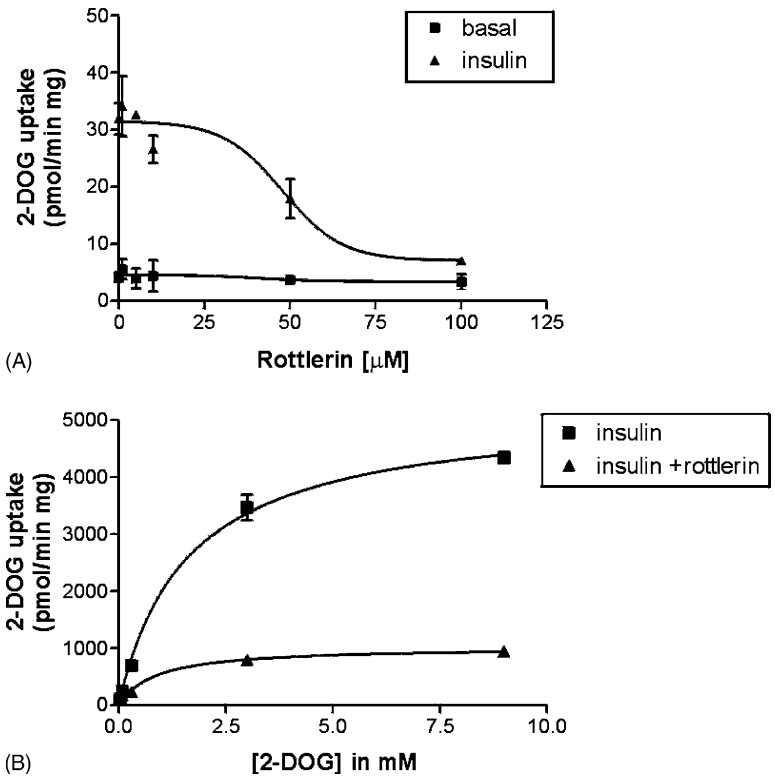

Fig. 5. A kinetic analysis of the effects of rottlerin on insulin-induced 2-DOG uptake. (Panel A) 3T3-L1 adipocytes were prestimulated with 100 nM insulin for 15 min as indicated. Subsequently, rottlerin as indicated was added concomitantly with the radiolabeled glucose in 30 μM 2-DOG and 5 min later glucose uptake was measured. Data shown are mean ± S.E. of an experiment performed in triplicate. (Panel B) 3T3-L1 adipocytes were prestimulated with insulin as in panel A and subjected to a kinetic analysis using the indicated concentrations of 2-DOG for 1 min. When indicated, the adipocytes were co-treated with 10 μM rottlerin added concomitantly with the radiolabeled glucose. Data shown are mean ± S.E. of an experiment performed in triplicate.

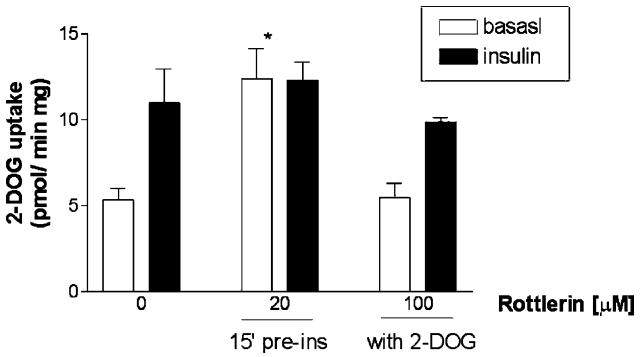

Fig. 6. Effects of rottlerin on insulin-induced glucose uptake in 3T3-L1 fibroblasts. 3T3-L1 fibroblasts were pretreated with 20 μM rottlerin for 15 min, or with 100 μM concomitant with the addition of the radiolabeled glucose. The fibroblasts were subjected to stimulation with 100 nM insulin for 15 min as indicated and assayed for [2-*deoxy*-D-^{14}C]glucose (2-DOG) uptake with 30 μM 2-DOG. Values are mean ± S.E. of two determinations performed in duplicate. An asterisk (*) indicates $P < 0.05$ as compared to untreated basal samples.

3.6. Effects of rottlerin on insulin-induced glucose uptake in 3T3-L1 fibroblasts

Given that rottlerin treatment did not affect GLUT1 translocation and did not affect basal levels of glucose uptake in 3T3-L1 adipocytes, it can be suggested that rottlerin acts specifically on the GLUT4 transporter and not on the GLUT1 transporters. To determine this, we analyzed the effects of rottlerin on 3T3-L1 fibroblasts. In these cells, insulin induces a small increase in glucose uptake mediated through the activity of GLUT1, whereas they do not express GLUT4. In these cells rottlerin did not inhibit insulin-induced glucose uptake. Remarkably, however, rottlerin treatment of unstimulated fibroblasts for 15 min induces a level of glucose uptake as high as (and not additive to) that attained by insulin stimulation (see Fig. 6).

4. Discussion

In a recent manuscript, Kayali et al. reported that rottlerin acts as a pharmacological inhibitor of insulin-induced glucose uptake. Furthermore, they observed that rottlerin depletes intracellular levels of ATP and suggested that these events were causally linked. In this paper, we confirmed that rottlerin potently inhibited insulin-induced glucose uptake with an IC_{50} of 10 μM and reduced the intracellular ATP concentration in 3T3-L1 adipocytes by 80%. Moreover, it was observed that the adipocytes respond to this situation in vivo by activating AMPK, as is demonstrated by the induction of AMPK and ACC phosphorylation [20]. Importantly, in 3T3-L1 adipocytes AMPK does not contribute to GLUT4 translocation [22]. And indeed, no significant effect of rottlerin is observed on basal levels of GLUT4 (see Fig. 3A). Intriguingly, in whole cell lysates we also observed insulin-induced AMPK phosphorylation. Rather than strictly activating lipogenesis and inhibiting lipolysis, insulin has been suggested to induce a futile cycle, with the net effect being lipogenesis [23]. Our observations, though not formally proving this "futile cycle" theory, does suggest such may indeed be the case in insulin signaling in 3T3-L1 adipocytes.

As is shown in Fig. 3, the predominant cause of the reduction in glucose uptake in 3T3-L1 adipocytes is an inhibition of insulin-induced GLUT4 translocation towards the PM. GLUT4 (but not GLUT1) translocation is dependent on two signaling pathways, i.e. PI-3′ kinase activation and Cbl tyrosine phosphorylation (reviewed in Refs. [3–5]). The data in Fig. 4 exclude an effect of rottlerin on the former pathway. This observation is somewhat surprising as Kayali et al. [11] reported an effect on insulin-induced PKB and ERK phosphorylation. Given that we applied similar conditions and used identical materials, we do not have an explanation for this discrepancy. We also tested the effects of rottlerin on arsenite-induced glucose transport, which was previously shown by us to occur independent of PI-3′ kinase [24]. In these experiments, an identical IC_{50} value of 10 μM was found, suggesting that the target of rottlerin indeed resides outside the PI-3′ kinase signaling pathway (Fig. 1).

When analyzing Cbl phosphorylation we observed a marked attenuation of insulin-induced tyrosine phosphorylation, suggesting rottlerin inhibits GLUT4 translocation by interfering with this pathway. After insulin stimulation, Cbl is tyrosine phosphorylated by the activated IR tyrosine kinase [25,26]. It seems unlikely that the lack of Cbl tyrosine phosphorylation is directly caused by a reduction in intracellular ATP concentration interfering with the activity of the IR tyrosine kinase: because other phosphorylation activities of the insulin receptor (IR) tyrosine kinase domain, such as autophosphorylation and IRS tyrosine phosphorylation, are unaffected by rottlerin. Moreover, though the reduction of intracellular levels may appear severe (80%), it must be noted the levels of ATP in 3T3-L1 adipocytes are very high (normally around 10 mM). When estimating the intracellular levels of ATP after rottlerin treatment, an ATP concentration of 2.3 mM is calculated. Thus, after rottlerin treatment the intracellular concentration of ATP is still much higher than the reported K_m of 8 μM of the IR tyrosine kinase for ATP [27]. These considerations argue against a deleterious effect on the IR tyrosine kinase activity by the reduction of intracellular ATP levels. However, an indirect effect of reduced ATP levels in the 3T3-L1 adipocyte, such as par example activation of an associated tyrosine phosphatase capable of removing tyrosine phosphates from Cbl, are not ruled out by our observations.

Aside from its effects on GLUT4 translocation we observed that rottlerin could inhibit insulin-induced glucose uptake directly. In experiments wherein GLUT4 had already been fully embedded in the PM, rottlerin still inhibited glucose uptake significantly by 30%. Studies on

3T3-L1 fibroblasts which express GLUT1 but not GLUT4, demonstrated this effect was specific for the GLUT4 transporter (see Fig. 6). Intriguingly, rather than acting as an inhibitor, rottlerin was observed to stimulate glucose uptake in 3T3-L1 fibroblasts. A possible explanation for this intriguing phenomenon is provided by a study of Barnes et al. [28]. In this study, the authors demonstrate that AMPK is involved in activating GLUT1-mediated glucose uptake in clone9 cells. Thus, in 3T3-L1 fibroblasts a similar mechanism in response to rottlerin-induced ATP-depletion could be involved. Intriguingly, no such effect was observed on basal levels of glucose uptake in 3T3-L1 adipocytes, suggesting that whatever the pathway connecting AMPK with GLUT1, it is lost during differentiation into the mature adipocyte and the emplacement of the highly specialized insulin-responsive vesicular GLUT4 glucose uptake system. (See El Jack et al. for a study describing this phenomenon in detail [29].)

The direct effect of rottlerin on the GLUT4 glucose transporter displayed the characteristics of uncompetitive inhibition. Uncompetitive inhibition occurs when a pharmacological inhibitor binds preferably to a substrate-bound enzyme. In the mechanism of glucose uptake a possible way to visualize this is that upon binding glucose from the extracellular environment the GLUT4 transporter becomes locked in a state in which it is unable to further transport the glucose into the cytoplasm. A direct consequence of uncompetitive inhibition is that the higher the amount of substrate added, the more pronounced the effects of rottlerin become. Thus, whereas the IC_{50} of the direct effect of rottlerin was 50 μM under 30 μM 2-DOG the IC_{50} under 3 mM 2-DOG drops profoundly to around 5 μM. It is noteworthy to realize that in vivo levels of glucose are about 5 mM. Thus, in a physiological setting, this direct inhibition of glucose uptake by rottlerin becomes highly relevant indeed.

Kayali et al. demonstrated that rottlerin inhibits insulin-induced glucose uptake and concomitantly reduces intracellular ATP levels. This led to the suggestion that the reduction of ATP actually interferes with GLUT4 vesicle translocation towards the PM [11]. It must be observed that we also observe a reduction of cellular levels of ATP upon rottlerin treatment of 3T3-L1 adipocytes and demonstrate an inhibition of GLUT4 translocation. Thus, the data presented in this manuscript should not be interpreted as ''proof against'' but are rather aimed to caution against a too straightforward interpretation of the effects of rottlerin. With respect to ATP-depletion and its effects on glucose uptake in 3T3-L1 adipocytes, however, several conflicting data have been reported in literature. On one hand, Kayali et al. [11] and Hresko et al. [30] observe a close link between a reduction of cellular ATP levels mediated by rottlerin, DNP, FCCP or glucosamine and an inhibition of glucose uptake in 3T3-L1 adipocytes. In contrast, however, Ross et al. [31] and Kang et al. [14] fail to observe these effects in 3T3-L1 adipocytes after glucosamine treatment. And employing DNP treatment Bashan et al. [32] and Khayat et al. [12] report the opposite, i.e. a stimulation of glucose uptake. Furthermore, data presented by Robinson et al. suggested that ATP may actually be required for intracellular sequestration of the GLUT4 transporter in 3T3-L1 adipocytes, ATP-depletion inducing rather than inhibiting GLUT4 translocation [13].

In conclusion, this study illustrates the pharmacological inhibitor rottlerin affects multiple processes involved in insulin-stimulated glucose uptake. Aside from an effect on cellular ATP levels, rottlerin inhibits Cbl tyrosine phosphorylation and reduces the activity of the GLUT4 glucose transporter, providing alternative (though not mutually exclusive) explanations for the observed inhibition of glucose uptake.

Acknowledgments

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References

[1] Kotani K, Ogawa W, Matsumoto M, Kitamura T, Sakaue H, Hino Y, et al. Requirement of atypical protein kinase C lambda for insulin stimulation of glucose uptake but not for Akt activation in 3T3-L1 adipocytes. Mol Cell Biol 1998;18:6971–82.
[2] Bandyopadhyay G, Standaert ML, Sajan MP, Kanoh Y, Miura A, Braun U, et al. PKC-{lambda} knockout in embryonic stem cells and adipocytes impairs insulin-stimulated glucose transport. Mol Endocrinol 2004;18:373–83.
[3] Elmendorf JS. Signals that regulate GLUT4 translocation. J Membr Biol 2002;190:167–74.
[4] Mora S, Pessin JE. An adipocentric view of signaling and intracellular trafficking. Diabet Metab Res Rev 2002;18:345–56.
[5] Bryant NJ, Govers R, James DE. Regulated transport of the glucose transporter GLUT4. Nat Rev Mol Cell Biol 2002;3:267–77.
[6] Inoue M, Chang L, Hwang J, Chiang SH, Saltiel AR. The exocyst complex is required for targeting of Glut4 to the plasma membrane by insulin. Nature 2003;422:629–33.
[7] Kanzaki M, Pessin JE. Insulin signaling: GLUT4 vesicles exit via the exocyst. Curr Biol 2003;13:R574–6.
[8] Gschwendt M, Muller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G, et al. Rottlerin, a novel protein kinase inhibitor. Biochem Biophys Res Commun 1994;199:93–8.
[9] Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 2000;351:95–105.
[10] Soltoff SP. Rottlerin is a mitochondrial uncoupler that decreases cellular ATP levels and indirectly blocks protein kinase C delta tyrosine phosphorylation. J Biol Chem 2001;276:37986–92.
[11] Kayali AG, Austin DA, Webster NJG. Rottlerin inhibits insulin-stimulated glucose transport in 3T3-L1 adipocytes by uncoupling mitochondrial oxidative phosphorylation. Endocrinology 2002;143:3884–96.
[12] Khayat ZA, Tsakiridis T, Ueyama A, Somwar R, Ebina Y, Klip A. Rapid stimulation of glucose transport by mitochondrial uncoupling depends in part on cytosolic Ca^{2+} and cPKC. Am J Physiol Cell Physiol 1998;44:C1487–97.

[13] Robinson LJ, Pang S, Harris DS, Heuser J, James DE. Translocation of the glucose transporter (GLUT4) to the cell surface in permeabilized 3T3-L1 adipocytes: effects of ATP insulin. J Cell Biol 1992;117: 1181–96.
[14] Kang J, Heart E, Sung CK. Effects of cellular ATP depletion on glucose transport and insulin signaling in 3T3-L1 adipocytes. Am J Physiol Endocrinol Metab 2001;280:E428–35.
[15] Telting D, van der Zon GC, Dorrestijn J, Maassen JA. IRS-1 tyrosine phosphorylation reflects insulin-induced metabolic and mitogenic responses in 3T3-L1 pre-adipocytes. Arch Physiol Biochem 2001; 109:52–62.
[16] Reed BC, Lane MD. Insulin receptor synthesis and turnover in differentiating 3T3-L1 preadipocytes. Proc Natl Acad Sci USA 1980;77:285–9.
[17] Van den Berghe N, Ouwens DM, Maassen JA, van Mackelenbergh MG, Sips HC, Krans HM. Activation of the Ras/mitogen-activated protein kinase signaling pathway alone is not sufficient to induce glucose uptake in 3T3-L1 adipocytes. Mol Cell Biol 1994; 14:2372–7.
[18] Simpson IA, Yver DR, Hissin PJ, Wardzala LJ, Karnieli E, Salans LB, et al. Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: characterization of subcellular fractions. Biochim Biophys Acta 1983;763:393–407.
[19] Bazuine M, Ouwens DM, Gomes de Mesquita DS, Maassen JA. Arsenite stimulated glucose transport in 3T3-L1 adipocytes involves both Glut4 translocation and p38 MAPK activity. Eur J Biochem 2003;270:3891–903.
[20] Hardie DG, Pan DA. Regulation of fatty acid synthesis and oxidation by the AMP-activated protein kinase. Biochem Soc Trans 2002; 30:1064–70.
[21] Holman GD, Lo-Leggio L, Cushman SW. Insulin-stimulated GLUT4 glucose transporter recycling. A problem in membrane protein subcellular trafficking through multiple pools. J Biol Chem 1994;269: 17516–24.
[22] Sakoda H, Ogihara T, Anai M, Fujishiro M, Ono H, Onishi Y, et al. Activation of AMPK is essential for AICAR-induced glucose uptake by skeletal muscle but not adipocytes. Am J Physiol Endocrinol Metab 2002;282:E1239–44.
[23] Hardie DG, Carling D, Carlson M. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu Rev Biochem 1998;67:821–55.
[24] Bazuine M, Ouwens DM, Gomes de Mesquita DS, Maassen JA. Arsenite stimulated glucose transport in 3T3-L1 adipocytes involves both Glut4 translocation and p38 MAPK activity. Eur J Biochem 2003;270:3891–903.
[25] Baumann CA, Ribon V, Kanzaki M, Thurmond DC, Mora S, Shigematsu S, et al. CAP defines a second signalling pathway required for insulin-stimulated glucose transport. Nature 2000;407:202–7.
[26] Liu J, Kimura A, Baumann CA, Saltiel AR. APS facilitates c-Cbl tyrosine phosphorylation and GLUT4 translocation in response to insulin in 3T3-L1 adipocytes. Mol Cell Biol 2002;22:3599–609.
[27] Bak JF, Handberg A, Beck-Nielsen H, Pedersen O. Kinetics of insulin binding and kinase activity of the partially purified insulin receptor from human skeletal muscle. Biochim Biophys Acta 1990;1052: 306–12.
[28] Barnes K, Ingram JC, Porras OH, Barros LF, Hudson ER, Fryer LG, et al. Activation of GLUT1 by metabolic and osmotic stress: potential involvement of AMP-activated protein kinase (AMPK). J Cell Sci 2002;115:2433–42.
[29] El Jack AK, Kandror KV, Pilch PF. The formation of an insulin-responsive vesicular cargo compartment is an early event in 3T3-L1 adipocyte differentiation. Mol Biol Cell 1999;10:1581–94.
[30] Hresko RC, Heimberg H, Chi MM, Mueckler M. Glucosamine-induced insulin resistance in 3T3-L1 adipocytes is caused by depletion of intracellular ATP. J Biol Chem 1998;273:20658–68.
[31] Ross SA, Chen X, Hope HR, Sun S, McMahon EG, Broschat K, et al. Development and comparison of two 3T3-L1 adipocyte models of insulin resistance: increased glucose flux vs. glucosamine treatment. Biochem Biophys Res Commun 2000;273:1033–41.
[32] Bashan N, Burdett E, Guma A, Sargeant R, Tumiati L, Liu Z, et al. Mechanisms of adaptation of glucose transporters to changes in the oxidative chain of muscle and fat cells. Am J Physiol 1993;264:C430–40.