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Selective cyclooxygenase-2 inhibitors stimulate glucose transport in L6 myotubes in a protein kinase Cδ-dependent manner

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ABSTRACT

Selective inhibitors of cyclooxygenase-2 (prostaglandin-endoperoxide synthase-2; COX-2) augment the rate of hexose uptake in myotubes by recruiting glucose transporter-4 (GLUT-4) to the plasma membrane in an insulin- and AMPKα-independent manner [Alpert E, Gruzman A, Lardi-Studler B, Cohen G, Reich R, Sasson S. Cyclooxygenase-2 (PTGS2) inhibitors augment the rate of hexose transport in L6 myotubes in an insulin- and AMPKα-independent manner. Diabetologia 2006;49:562–70]. We aimed at elucidating the molecular interactions that mediate this effect of COX-2 inhibitors in L6 myotubes. The effects of the inhibitors niflumic acid, nimesulide and rofecoxib on activities and phosphorylation state of key proteins in the insulin transduction pathway were determined. These inhibitors did not induce specific tyrosine phosphorylation in IRS-1, could not assemble a functional IRS-PI3K-PKB/Akt complex and did not activate GSK3α/β, JNK1/2, ERK1/2, p38-MAPK or c-Cbl by site-specific phosphorylation(s). Yet, like insulin, they activated mTOR and induced downstream threonine phosphorylation in p70S6K and 4EBP1. However, rapamycin, which inhibits mTOR enzymatic activity, did not interfere with COX-2 inhibitor-induced stimulation of hexose uptake in myotube. Thus, mTOR activation was not required for COX-2 inhibitor-dependent augmentation of hexose transport in myotubes. Because PKCδ has also been shown to activate mTOR, we asked whether COX-2 inhibitors activate mTOR by a prior activation of PKCδ. Indeed, all three inhibitors induced tyrosine phosphorylation in PKCδ and stimulated its kinase activity. Moreover, pharmacological inhibition of PKCδ or the expression of a dominant-negative form of PKCδ in myotubes completely abolished COX-2 inhibitor-dependent stimulation of hexose uptake. This study shows that selective COX-2 inhibitors activate a unique PKCδ-dependent pathway to increase GLUT-4 abundance in the plasma membrane of myotubes and augment the rate of hexose transport.

1. Introduction

Selective cyclooxygenase-2 (COX-2; prostaglandin endoperoxide synthase-2) inhibitors may cause hypoglycemic episodes in man when over-consumed or in a combination therapy with oral antihyperglycemic drugs ([1] and references therein). We have shown recently that some selective COX-2 inhibitors (niflumic acid, nimesulide and rofecoxib) augment

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the rate of hexose transport in L6 myotubes in a dose- and time-dependent manner by increasing the plasma membrane abundance of the insulin-sensitive glucose transporter-4 (GLUT-4) but not of the ubiquitously expressed GLUT-1 [1]. In contrast, COX-1 inhibitors (i.e., acetyl salicylic acid and indomethacin) had no effect on the hexose transport system in L6 myotubes [1]. The translocation of GLUT-4 to the plasma membrane of skeletal muscle usually occurs following the activation of the insulin receptor-dependent transduction pathway or through the 5′-AMP-activated kinase (AMPKα)-dependent pathway in contracting muscles [2]. We have shown that the hexose transport stimulatory effect of these inhibitors is slow (6-h lag period; maximal effect at 12 h), insulin-independent and does not entail AMPKα activation [1]. In the present study, we addressed the hypothesis that COX-2 inhibitors increase the abundance of GLUT-4 in the plasma membrane in myotubes by circumventing the initial step of insulin transduction pathway and activating downstream transducer proteins or by utilizing other unrelated mechanisms.

Insulin-induced translocation of GLUT-4-containing vesicles to the plasma membrane of myotubes occurs upon the binding of the hormone to its cell-surface receptor, induction of tyrosine kinase activity in its β-subunit and activation of a downstream phosphorylation cascade that assembles and activates regulatory complexes [2]. The initial step in this cascade is the phosphorylation of certain tyrosine residues in insulin receptor substrates (IRS) and the formation of insulin receptor-IRS docking complexes for various regulatory proteins with SH2 domain. Both IRS-1 and IRS-2 are expressed in L6 myotube. The former is considered the predominant isoform that mediates GLUT-4 translocation to the plasma membrane in skeletal muscles [2]. Several interactions that enhance the rate of IRS-1 degradation slow or terminate insulin action have been proposed: for instance, glycogen synthase kinase-3 (GSK3α/β)-dependent phosphorylation of target serine residues in IRS-1 directs it to proteasomal degradation. The inactivation of GSK3α/β by serine^{21}- and serine^{9} phosphorylation, respectively, may slow IRS-1 degradation and extend the duration of insulin action [3,4]. The role of IRS-2 in regulating glucose transport in L6 myotubes is ambiguous as silencing of IRS-1 expression, but not of IRS-2, reduced insulin-dependent GLUT-4 translocation to the plasma membrane [5]. The insulin receptor-IRS-1 complex activates PI3-kinase, whose metabolite phosphatidylinositol (PI)-3,4,5-trisposphate then recruits 3-phosphoinositide-dependent protein kinase-1 (PDK1). The latter activates protein kinase B (PKB/Akt) by targeted serine/threonine phosphorylations, allowing it to phosphorylate other protein kinases and regulatory proteins, which mediate a variety of cellular processes, including the translocation of GLUT-4-containing vesicles to the plasma membrane [2].

Insulin-induced targeted tyrosine phosphorylation of c-Cbl has been linked to an enhanced recruitment of GLUT-4-containing vesicles to the plasma membrane in 3T3L1 adipocytes [6,7]. Notwithstanding the dispute whether this pathway functions in insulin-treated adipocytes [8], it might contribute to GLUT-4 translocation in skeletal muscles [9], possibly due to the expression of a novel skeletal muscle-specific c-Cbl associated protein [10]. Members of the MAPK family are also activated by insulin: JNK1/2 facilitate the inactivation of IRS-1 [11]. The intrinsic activity of GLUT-4 might be regulated by p38-MAPK [12]. ERK1/2 have also been linked to insulin signaling in skeletal muscles [13]. The active PI3K-PKB/Akt complex also activates mammalian target of rapamycin (mTOR), which regulates various downstream pathways, such as augmentation of rate of translation of a family of mRNAs that encode components of the protein synthesis machinery or ensues cap-dependent translation initiation by activating p70S6K and 4EBP1, respectively [14,15]. Sampson and colleagues have recently identified a role for PKCδ in insulin action in skeletal muscle cells: myotubes overexpressing a kinase inactive, dominant negative PKCδ became resistant to insulin-induced stimulation of glucose uptake and GLUT-4 translocation, while overexpression of PKCδ increased the rate of glucose uptake, even in the absence of insulin [16].

We used niflumic acid, nimesulide and rofecoxib that represent early and newer generations of selective COX-2 inhibitors [1]. While there is no indication for a COX-2 inhibitor-induced activation of key elements in the classical insulin transduction mechanism, this study shows that these inhibitors stimulate the glucose transport system in myotubes in a unique PKCδ-dependent manner.

2. Materials and methods

2.1. Materials

The sources of materials used in this study were as follows: αMEM and FCS from Biological Industries (Beth-Haemek, Israel); 2-[1,2-^{3}H(N)]-deoxy-D-glucose (2.22 TBq/mmol) from American Radiolabeled Chemicals (St. Louis, MO, USA); 2-deoxy-D-glucose (dGlc), LY294,002, niflumic acid, nimesulide and rottlerin from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Rapamycin from Calbiochem (La Jolla, CA, USA); Protein G- and protein A Sepharose™ beads were from Amersham Pharmacia Biothech (Uppsala, Sweden). Rofecoxib was kindly donated by Merck Research Laboratories (Rahway, NJ, USA). All other chemicals, reagents and solvents were reagent- or molecular biology-grade. The antibodies used were against: IRS-1, IRS-2, phospho-S^{307}-IRS-1, phospho-Y (clone 4G10), PKB/Akt1 (PH domain), phospho-S^{473}-Akt1/PKBα and Cbl (clone 7G10) from Upstate Biotechnology (Lake Placid, NY, USA); phospho-Y^{774}-c-Cbl from Cell Signaling Technology (Beverly, MA, USA); phospho-T^{308}-PKB/Akt1/2/3, PKCδ (C-17), p70S6K, phospho-T^{389}-p70S6K, phospho-T^{69}-4EBP1, phospho-S^{21}-GSK3α and phospho-S^{9}-GSK3β from Santa Cruz Biotechnology (Santa Cruz, CA, USA); phospho-T/Y$^{185/187}$-ERK1/2, phospho-T/Y$^{183/185}$-JNK1/2 and phospho-T/Y$^{180/182}$-p38-MAPK from Biosource International (Camarillo, CA, USA).

2.2. Cell cultures

L6 skeletal myocytes were grown and let to differentiate into multi-nuclear myotubes (85–90% yield) in αMEM supplemented with 2% (v/v) FCS, as described [17].

2.3. Hexose uptake assay

The [^{3}H]-2-deoxy-D-glucose ([^{3}H]-dGlc) uptake assay in L6 myotubes, in the absence or presence of insulin (100 nmol/l, 20 min) was performed as described [1,18]. COX-2 inhibitors (dissolved in DMSO) or other compounds (dissolved in ethanol or DMSO) were added to cell cultures from stocks solutions by 1000-dilution. DMSO reduced the rate of hexose transport 3–5%, while ethanol had no such effects.

2.4. Preparation of cell lysates and Western blot analyses

Myotube cultures were lysed for 30 min in an ice-cold lysis buffer [in mmol/l: 50 Tris–HCl, pH 7.5, 1 EDTA, 1 EGTA, 1 Na_3VO_4, 50 NaF, 10 sodium β-glycerophosphate, 5 sodium pyrophosphate and 1 PMSF, and supplemented with 0.1% (v/v) Triton X-100, 0.1% (v/v) 2-β-mercaptoethanol and protease inhibitor cocktail (1:100 dilution)] and centrifuged (12,000 rpm in an Eppendorf centrifuge, 10 min at 4 °C). Protein content in the supernatant was determined according to Bradford, using BSA standard, dissolved in the same buffer. Aliquots (5–60 μg protein) were mixed with sample buffer [62.5 mmol/l Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 50 mmol/l DTT and 0.1% (w/v) bromophenol blue], heated at 95 °C for 5 min. Protein were separated by SDS/PAGE and Western blot analyses were performed, using commercial antibodies, according to the antibody supplier's protocol. Total cell content and plasma-membrane localised GLUT-4 were determined as described [1].

2.5. Immunoprecipitation

Aliquots of cell lysates (1 mg protein) were mixed with 25 μl protein A/G Sepharose™ beads and incubated and rotated for 30 min at 4 °C. Following centrifugation (12,000 rpm, in an Eppendorf centrifuge, 10 min at 4 °C), the supernatant was added to 30 μl of fresh protein A/G Sepharose™ beads together with the proper antibody (2–4 μg) and left rotating overnight at 4 °C. The pellet was then washed twice by centrifugation in the lysis buffer. The bead pellet was resuspended in 50 μl of RIPA buffer [in mmol/l: 50 Tris–HCl, pH 7.4, 150 NaCl, 1 EDTA and 1 PMSF, supplemented with 0.1 (w/v) SDS, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate and protease inhibitor cocktail], heated at 95 °C for 5 min and used for SDS-PAGE and Western blot analyses.

2.6. Recombinant adenovirus constructs and viral infection of cultures

Recombinant adenovirus vectors harboring the gene for β-galactosidase or kinase inactive, dominant negative mouse PKCδ were constructed and used as described [19]. The latter was generated by substitution of the lysine residue at the ATP binding site with alanine [20,21]. L6 myotube cultures were rinsed and infected with a viral supernatant in fresh complete αMEM and incubated for 1 h. The cultures were then washed twice with αMEM and incubated in complete culture medium for additional 24 h, followed by treatments with COX-2 inhibitors and/or insulin. A staining kit for β-galactosidase in fixed cells (Imgenex, San Diego, CA) was used according to the manufacturer's protocol to determine infection efficiency.

2.7. In vitro PKCδ enzymatic assay

The kinase assay was performed as previously described [22]. In brief, post-nuclear lysates of myotubes were prepared and PKCδ was immunoprecipitated with an anti-PKCδ antibody. The kinase activity in the eluates was measured with the SingaTECT PKC assay kit (Promega, Madison, WI) according to the manufacturer's protocol, using PKCα pseudosubstrate.

2.8. Statistical analysis

Data are given as means ± S.E.M.; Student's two-tailed *t*-test was used for group comparisons. A p value of <0.05 was taken to indicate statistical significance.

3. Results

3.1. COX-2 inhibitors augmented the rate of hexose uptake and GLUT-4 abundance in the plasma membrane of L6 myotubes

The stimulatory effects of COX-2 inhibitors on the rate of hexose uptake and GLUT-4 abundance in the plasma membrane of L6 myotubes are summarised in Fig. 1. Exposure of myotubes for 16 h to nimesulide, niflumic acid and rofecoxib augmented the rate of [^{3}H]-2-deoxy-D-glucose (dGlc) uptake 2.6 ± 0.15- 2.39 ± 0.17- and 2.15 ± 0.19-fold and increased GLUT-4 content in the plasma membrane 2.01 ± 0.14-, 1.86 ± 0.18- and 1.73 ± 0.17-fold, respectively, in comparison with control myotubes. Insulin (20 min) was less potent than COX-2 inhibitors and increased the rate of dGlc uptake and GLUT-4 content in the plasma membrane 1.57 ± 0.10- and 1.45 ± 0.07-fold, respectively. These results are consistent with our previous data on the independent and additive effects of these COX-2 inhibitors and insulin on the hexose transport system in L6 myotubes [1]. Total cell content and plasma membrane abundance of the ubiquitous GLUT-1 and GLUT-3 in L6 myotubes were not altered in the presence of these inhibitors [1]. The optimal effective concentrations used (200 μmol/l of nimesulide or niflumic acid and 300 μmol/l of rofecoxib) and the optimal length of the incubation period (16 h) were determined before [1].

3.2. COX-2 inhibitors failed to activate IRS-1, PKB/Akt or PI3K, but induced IRS-2 phosphorylation

We first asked whether COX-2 inhibitors could mimic insulin action and recruit GLUT-4 to the plasma membrane of L6 myotubes by activating IRS-1 or IRS-2 in a non-insulin-dependent manner (Fig. 2A). As expected, insulin rapidly induced substantial tyrosine phosphorylation in IRS-1, which decayed within 20 min concomitant with a pronounced serine^{307}-phosphorylation. In contrast, no such tyrosine- or serine phosphorylations in IRS-1 were observed in niflumic acid-, nimesulide- and rofecoxib-treated myotubes. In addition, insulin-induced tyrosine- and serine-phosphorylations

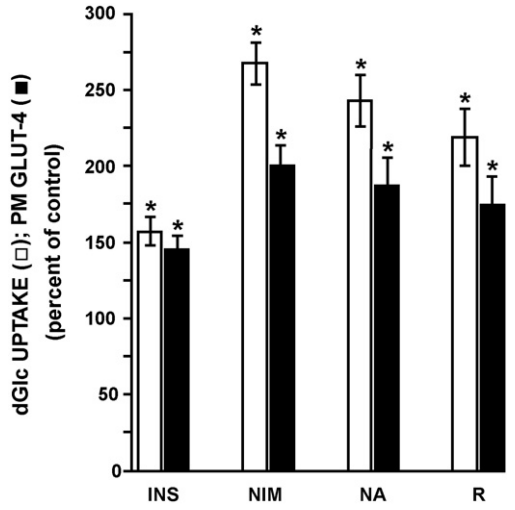

Fig. 1 – COX-2 inhibitors augment the rate of hexose transport and GLUT-4 content in the plasma membrane of L6 myotubes. Confluent myotubes cultures that had been maintained at 23.0 mmol/l glucose for 24 h were exposed to 200 μmol/l of nimesulide (NIM) or niflumic acid (NA) or 300 μmol/l of rofecoxib (R) for additional 16 h. Some cultures were treated with insulin (INS, 100 nmol/l) during the last 20 min of incubation. The rates of dGlc uptake (open bars, $n = 6$) were measured and cell surface-localised GLUT-4 (black bars, $n = 3–4$) was determined by the cell surface biotinylation procedure, as described [1]. The basal rate of dGlc uptake (1.01 ± 0.12 nmol/mg protein/min) and the density of the GLUT-4 band of control myotubes, incubated at 23.0 mmol/l glucose, were taken as 100% values. Mean ± S.E.M., $p < 0.05$, *in comparison with each 100% respective control. Final glucose concentrations at the end of the last incubation period were reduced to ~20 and ~17 mM in culture media of control and COX2 inhibitors-treated cells, respectively.

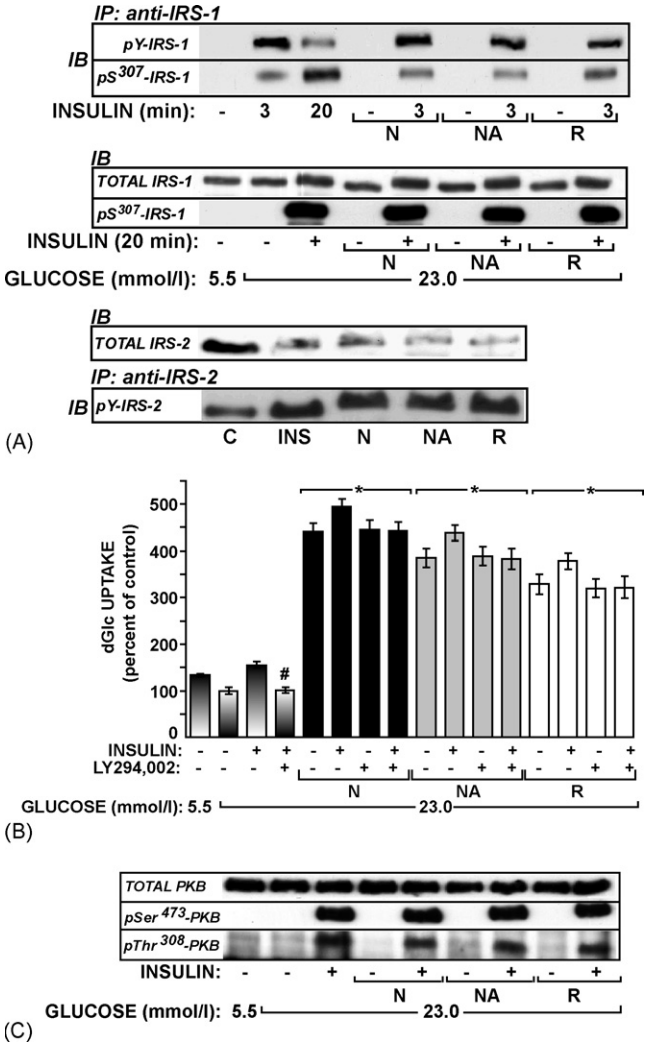

Fig. 2 – COX-2 inhibitors act in an IRS-1-, PI3K- and PKB/Akt-independent manner, but induce tyrosine phosphorylation in IRS-2. L6 myotubes were treated with nimesulide (N), niflumic acid (NA) or rofecoxib (R) and/or insulin as described in the legend to Fig. 1. Cell lysates were prepared, immunoprecipitated, separated by SDS-PAGE and used for the following Western blot analyses. (A) Total IRS-1 and IRS-2 in cell lysates were detected with the proper antibodies; pY-IRS-1, pY-IRS-2 and pS^{307}-IRS-1 were immunoblotted with an anti-pY antibody (clone 4G10) or anti-pS^{307}-IRS-1 antibody, respectively, following immunoprecipitation with anti-IRS-1 or anti-IRS-2 antibodies. The M_r of IRS-1 and IRS-2 were ~170–180 kDa. (B) L6 myotubes were treated as described above. LY292,202 (60 μmol/l) was added 10 min before the addition of COX-2 inhibitors or insulin. The rate dGlc uptake was measured at the end of incubation, and that measured in control myotubes (1.12 ± 0.09 nmol/mg protein/min) was taken as 100%. Mean ± S.E.M., $n = 3$, $p < 0.05$, in comparison with #insulin-treated myotubes or *control and insulin-treated myotubes. (C) Lysates of myotubes, which had been treated with COX-2 inhibitors and/or insulin, as described above, were separated by SDS-PAGE and analyzed by Western blotting for total PKB/Akt, pS^{473}- and pT^{308}-PKB/Akt1, using specific antibodies.

of IRS-1 were not altered in the presence of these inhibitors. Surprisingly, the three inhibitors mimicked insulin and induced prominent tyrosine phosphorylation in IRS-2 (1.74 ± 0.19-, 2.03 ± 0.26-, 2.17 ± 0.39-, and 2.07 ± 0.42-fold increase following treatments with insulin, nimesulide, niflumic acid and rofecoxib, respectively, in comparison with control myotubes. Means ± S.E.M., $n = 3$). Equally interesting, an apparent mobility shift of IRS-2, coupled with a distinct reduction in its content, was apparent in COX-2 inhibitor-treated myotubes.

The PI3K inhibitor LY294002 (60 μmol/l) was used to determine whether active PI3K was required for COX-2 inhibitor-induced stimulation of hexose uptake (Fig. 2B). As expected, LY294002 blocked completely the stimulatory effect of insulin. However, the hexose transport stimulatory effects of COX-2 inhibitors remained unaltered and high in the presence of LY294002 whether it was present throughout the entire 16-h incubation period (Fig. 2B) or when it was added at 4-, 8-, and 12 h within it (data not shown). Fig. 2B also shows that in myotubes treated with both COX-2 inhibitor and insulin, LY294002 effectively abolished only the fraction of

insulin-dependent stimulation of hexose transport, leaving the fraction induced by niflumic acid, nimesulide or rofecoxib intact.

Treatment of L6 myotubes with insulin resulted also in the phosphorylation of serine^{473} and threonine^{308} residues in PKB/Akt (Fig. 2C). However, none of the COX-2 inhibitors induced a similar pattern of phosphorylation in PKB/Akt, nor did they interfere with that induced by insulin. The total cell content of PKB/Akt remained unaltered in COX-2 inhibitor- and/or high glucose treated myotubes.

3.3. *COX-2 inhibitors did not induce site-specific phosphorylation in GSK3α/β, ERK1/2, JNK1/2, p38-MAPK or c-Cbl*

PKB/Akt-dependent phosphorylation of GSK3α/β attenuates their capacity to phosphorylate serine residues in IRS-1 and target it to proteasomal degradation [3,4]. Two observations ruled out the assumption that COX-2 inhibitors augmented the rate hexose transport in myotubes by inactivating GKK3α/β, followed by a slower rate of degradation of IRS-1. First, the cellular content of IRS-1 was not altered in COX-2 inhibitor-treated myotubes (Fig. 2A). Second, unlike insulin, COX-2 inhibitors did not inactivate GKK3α/β by inducing serine^{21}- and serine^{9}-phosphotylation, respectively (Fig. 3A).

Fig. 3B depicts a characteristic pattern of insulin-induced phosphorylation of threonine and tyrosine moieties in JNK1/2, ERK1/2 and p38MAPK. The capacity of insulin to induce tyrosine^{774} phosphorylation in c-Cbl in myotubes is depicted in Fig. 3C. However, nimesulide, niflumic acid and rofecoxib failed to induce such specific patterns of phosphorylations nor did they modify insulin-dependent phosphorylations of JNK1/2, ERK1/2 and p38MAPK (Fig. 3B) or c-Cbl (Fig. 3C).

3.4. *COX-2 inhibitors activate mTOR in L6 myotubes*

Upon insulin stimulation, PKB/Akt also activates mTOR, which subsequently mediates the phosphorylation of p70S6K and 4EBP1 [14,15]. A COX-2 inhibitor-dependent activation of mTOR seemed unlikely, because there was no indication that the inhibitors promoted the assembly of a functional PI3K-PKB/Akt pathway (Fig. 2). Nevertheless, Fig. 4A shows that nimesulide, niflumic acid and rofecoxib induced threonine^{389}- and threonine^{69}-phosphorylation in p70S6K and 4EBP1, respectively. These effects were mediated by mTOR, since the mTOR inhibitor rapamycin abolished both insulin- and COX-2 inhibitor-dependent phosphorylation of both target proteins. Strikingly, inhibition of mTOR by rapamycin, which attenuated insulin-dependent stimulation of hexose transport, left the COX-2 inhibitor-dependent stimulation of hexose uptake in myotubes intact (Fig. 4B). Thus, the two effects of selective COX-2 inhibitors in L6 myotubes – activation of mTOR, on one hand, and augmentation of hexose transport, on the other – seem independent.

3.5. *PKCδ mediated COX-2 inhibitors-dependent stimulation of hexose uptake in L6 myotubes*

Based on previous observations that PKCδ activates mTOR in a PI3K-independent manner [22–24] and our findings that COX-2 inhibitor-induced activation of mTOR was not associated with the hexose uptake stimulatory effect of the inhibitors, we

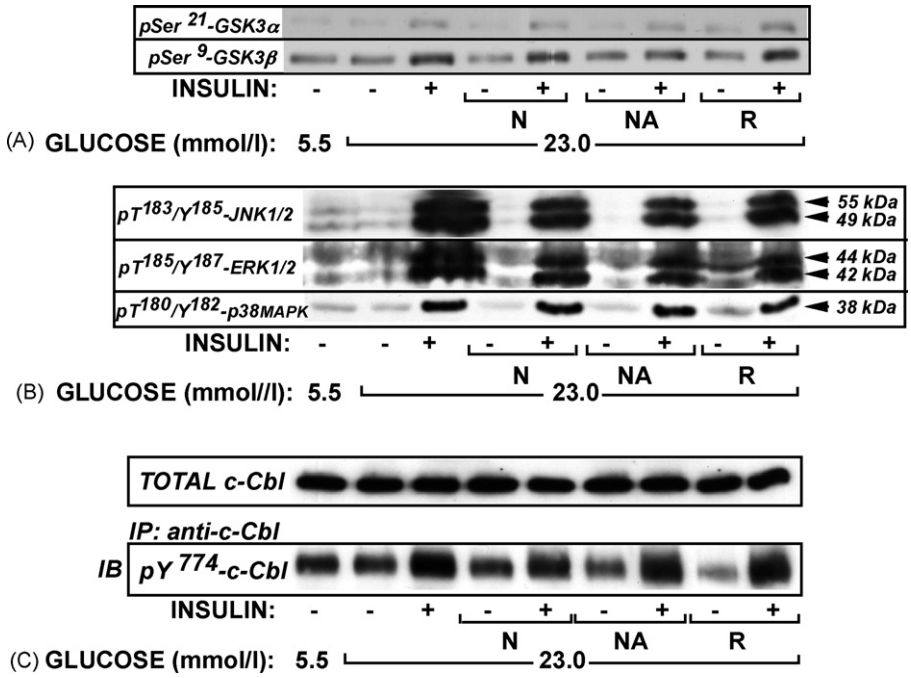

Fig. 3 – COX-2 inhibitors do not induce site-specific phosphorylations in GSK3α/β, JNK1/2, ERK1/2, p38-MAPK or c-Cbl. Lysates were prepared from L6 myotubes that had been treated with nimesulide (N), niflumic acid (NA) or rofecoxib (R) without or with insulin, as described in the legend to Fig. 1, and used for Western blot analyses of: (A) pS^{21}-GSK3α (51 kDa), pS^{9}-GSK3β (46 kDa). (B) pTpY$^{183/185}$-JNK1/2, pTpY$^{185/187}$-ERK1/2 and pTpY$^{180/182}$-p38-MAPK. (C) Total c-Cbl (120 kDa) and pY^{774}-c-Cbl (the latter was blotted following immunoprecipitation with an anti c-Cbl antibody).

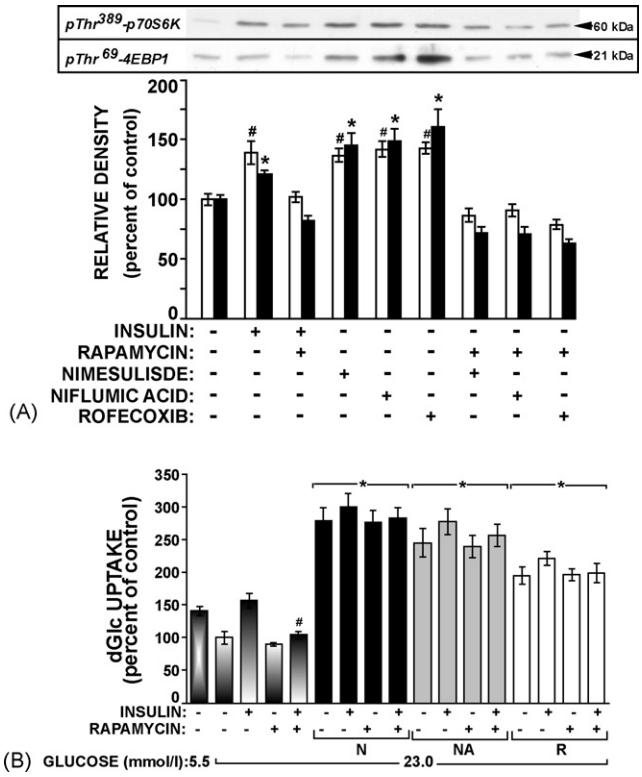

Fig. 4 – COX-2 inhibitors activate mTOR independently of their glucose transport augmenting activity. (A) L6 myotubes that had been maintained at 23.0 mmol/l glucose for 24 h were incubated without or with nimesulide, niflumic, rofecoxib and insulin as described in the legend to Fig. 1. Rapamycin (50 nmol/l) was added 30 min before the addition of COX-2 inhibitors or insulin. Western blot analyses of pT^{389}-p70S6K (open columns) and pT^{69}-4EBP1 (21 kDa; black columns) were performed as described under "Section 2". Representative Western blots and a summary of three individual experiments are shown. Mean ± S.E.M., $p < 0.05$, in comparison with the levels of #pT^{389}-p70S6K or *pT^{69}-4EBP1 in lysates of control myotubes. (B) L6 myotubes were treated similarly and the rate of dGlc uptake was measured at the end of the incubation period. N: nimesulide; NA: niflumic acid; R: rofecoxib. The 100% value was assigned to the rate of dGlc uptake (0.96 ± 0.09 nmol/mg protein/min) in control myotubes. Mean ± S.E.M., $n = 3$. $p < 0.05$, in comparison with #insulin-treated myotubes or to *control or insulin-treated cells.

hypothesized that the primary effect of COX-2 inhibitors was to promote the activation of PKCδ. Indeed, we found that COX-2 inhibitors and insulin induced tyrosine phosphorylation of PKCδ (Fig. 5A and B). This was associated with an increased catalytic activity of the enzyme: Fig. 5C shows that nimesulide, niflumic acid, rofecoxib and insulin increased 2.1 ± 0.3-, 2.0 ± 0.3- and 2.4 ± 0.5- 1.4 ± 0.1-fold, respectively, the kinase activity of immunoprecipitated PKCδ in comparison with that prepared from control myotubes.

Rottlerin (5 μmol/l), a selective inhibitor of PKCδ [25], prevented both insulin- and COX-2 inhibitor-dependent phosphorylation of PKCδ (Fig. 5A and B). Concomitantly, it reduced significantly insulin-, nimesulide-, niflumic acid- and rofecoxib-induced stimulation of the rate of hexose uptake in myotubes (compare 1.19 ± 0.07, 1.07 ± 0.05, 1.36 ± 0.08 and 1.29 ± 0.11, respectively, in the presence of rottlerin, to 1.64 ± 0.06, 3.15 ± 0.08, 2.80 ± 0.09, and 2.37 ± 0.09 nmol dGlc/mg protein/min, respectively, in its absence. Means ± S.E.M., $n = 3$, $p < 0.05$ for each treatment in comparison with its rottlerin-free control). Fig. 5D confirms previous studies [22–24] on a functional association between PKCδ and mTOR, as rottlerin prevented both insulin and COX-2 inhibitor-induced threonine phosphorylation in p70S6K and 4EBP1.

It should be noted, however, that rottlerin is also a potent mitochondrial uncoupler that alters ATP content in cells [26,27]. Mitochondrial dysfunction often operates a compensatory mechanism to augment the expression of GLUT-1 and the rate of glucose transport in cells [28]. We found that a 12–14 h exposure period of L6 myotubes to 5 μmol/l rottlerin resulted in a 1.7-fold increase of GLUT-1 (but not GLUT-4) content and a 1.4–1.6-fold increase in the rate of hexose transport (data not shown). Cellular ATP content was also measured [29]: while none of the three COX-2 inhibitors used altered it significantly, rottlerin (5 μmol/l) increased it 1.75-fold within 14 h, in comparison with control myotubes that were similarly exposed to 23.0 mmol/l glucose. We speculate that the rise in ATP content reflects a stress reaction that augmented the expression of GLUT-1, followed by a compensatory ATP generation through the glycolytic pathway. Remarkably, no similar effects of rottlerin on GLUT-1 expression and ATP content were observed when it was added to myotube cultures together with niflumic acid, nimesulide or rofecoxib for the same period.

Due to the complex effects of rottlerin, we sought an alternative approach to elucidate the role PKCδ in mediating the effects of COX-2 inhibitors on the glucose transport system. Myotube cultures were infected with recombinant adenoviral constructs expressing kinase inactive (dominant negative) PKCδ or (control) β-galactosidase. Over 90% of myotubes infected with the latter virus were stained positively for β-galactosidase (data not shown). Western blot analysis (Fig. 6, inset) with a polyclonal antibody against PKCδ demonstrated a ~3-fold higher content of the protein upon the expression of the D/N-PKCδ in myotubes, in comparison with non-infected myotubes, or with myotubes expressing β-galactosidase. The hexose uptake stimulatory effect of COX-2 inhibitors was measured in control and infected myotubes (Fig. 6): D/N-PKCδ rendered COX-2 inhibitors inactive and attenuated the effect of insulin. COX-2 inhibitor-treated control myotubes, which expressed β-galactosidase, increased the rate of hexose uptake to the same extent as non-infected myotubes that were treated similarly.

4. Discussion

This study shows that COX-2 inhibitors upregulate the rate of hexose in L6 myotubes and that this effect is mediated by activating PKCδ. Unlike the rapid insulin-induced translocation of GLUT-4 to the plasma membrane, maximal COX-2

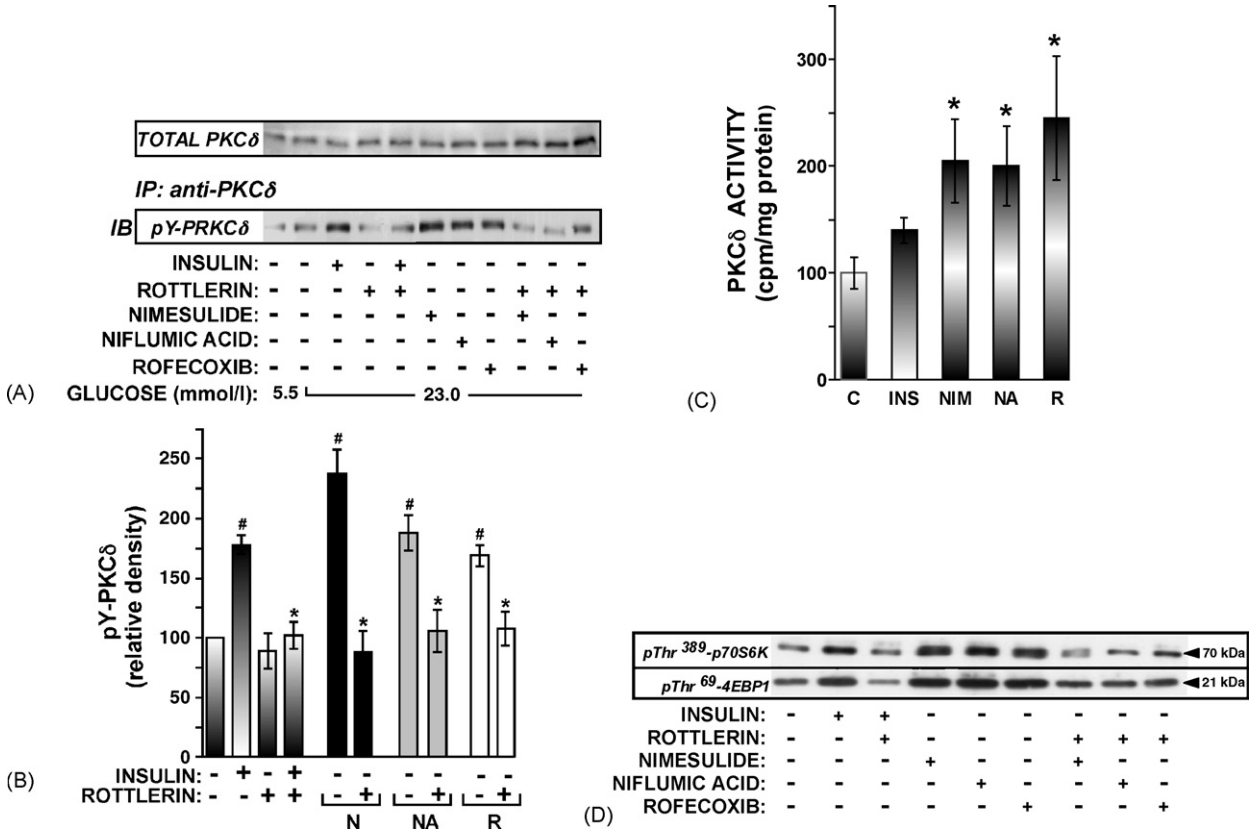

Fig. 5 – COX-2 inhibitors induce tyrosine phosphorylation and activate PKCδ. L6 Myotubes were treated with COX-2 inhibitors without or with insulin, as described in the legend to Fig. 1. Rottlerin (5 μmol/l) was added 30 min before the addition of nimesulide (N), niflumic acid (NA), rofecoxib (R) or insulin. At the end of the incubation period the myotubes were washed and lysates were prepared. (A) Western blot analysis of PKCδ (in cell lysates, 81 kDa) and of pY-PKCD (following immunoprecipitation with an anti-PKCδ antibody). (B) A summary of three independent Western blot analyses of pY-PKCδ. Mean ± S.E.M., $p < 0.05$, in comparison with #control myotubes or *nimesulide (N)-, niflumic acid (NA)- or rofecoxib (R)-treated myotubes. (C) In vitro kinase assay of immunoprecipitated PKCδ from lysates of insulin- and COX-2 inhibitor-treated myotubes. Mean ± S.E.M., $n = 3$. *$P < 0.05$, in comparison with immunoprecipitates prepared from control or insulin-treated cells. (D) Aliquots taken from the same lysates were analyzed by Western blotting for pT^{389}-p70S6K and pT^{69}-4EBP1.

inhibitor-induced recruitment of GLUT-4 to the plasma membrane required 12–16 h [1]. We showed before [1] that the combined effect of COX-2 inhibitors and insulin (added during the last 20 min of the 16-h exposure period to the inhibitors) on the rate of hexose transport was additive. The present study shows that the mechanisms of action of the inhibitors and insulin greatly differ: Fig. 2B shows that inhibition of PI3K abolished the effect of insulin, but left the inhibitors-dependent stimulation intact, indicating that the inhibitors did not assemble and utilize active regulatory complexes of the insulin transduction pathway (i.e., IRS-1, PI3K and PKB/Akt) to regulate the glucose transport system. Similarly, Fig. 2(A and C) and Fig. 3(A–C) show that GSK3α/β, c-Cbl, JNK1/2, ERK1/2 or p38-MAPK, which are activated by insulin, were not affected by any of the inhibitors. Our previous data [1] also rule out a COX-2-inhibitor dependent activation of AMPKα. Fig. 7 outlines the two independent pathways by which insulin and COX-2 inhibitors affect the subcellular distribution of GLUT-4 in myotubes.

Several agents (i.e., growth factors, hydrogen peroxide, cholinergic agonists) activate PKCδ by targeted phosphorylation of specific tyrosine residues [30]. Others have linked insulin-dependent activation of PKCδ to the stimulation of glucose uptake in skeletal muscles [31–33]. It has been suggested that insulin-dependent tyrosine phosphorylation of PKCδ is mediated by Src [16] and that a rapid upregulation of PKCδ expression occurs in insulin-treated skeletal muscle [34]. In contrast to these findings, other investigators have reported that the activation of PKCδ attenuates or even inhibits the tyrosine kinase activity of insulin receptor [35,36]. For instance, Greene et al. argue that active PKCδ inhibits insulin-stimulated IRS-1 tyrosine phosphorylation [37].

Substrate specificity and subcellular compartmentalisation of PKCδ are regulated by phosphorylation of distinct tyrosine residues, which promote its co-association with divergent binding/docking protein epitopes and substrates [38,39]. Thus, various pools of PKCδ, localised to distinct subcellular compartments, may mediate different, and occasionally contrasting

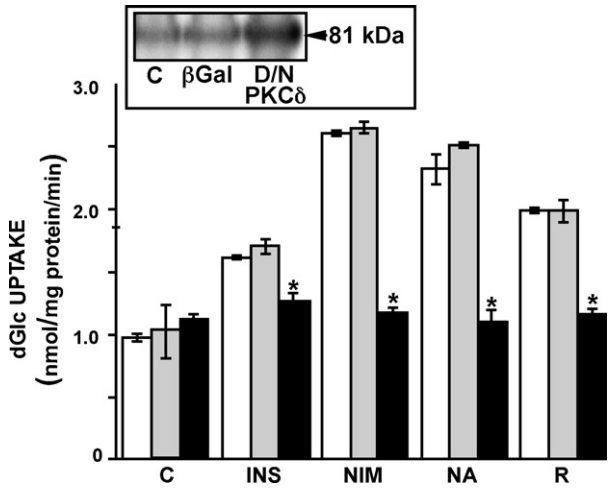

Fig. 6 – Expression of dominant negative-PKCδ prevents COX-2 inhibitor-induced stimulation of hexose uptake in L6 myotubes. Myotube cultures were infected with adenovirus vectors expressing D/N-PKCδ or β-galactosidase, as described under "Section 2". Control myotubes (open bars), β-galactosidase-expressing myotubes (gray bars) and D/N-PKCδ expressing myotubes (black bars) were then treated with nimesulide (N), niflumic acid (NA) or rofecoxib (R), as described in the legend to Fig. 1, and taken for the dGlc uptake assay. Mean ± S.E.M., $n = 3$, $^{*}p < 0.05$, in comparison with control myotubes or β-galactosidase-expressing myotubes, for each treatment. *Inset*: Western blot analysis of PKCδ in control (C), β-galactosidase- and D/N-PKCδ expressing myotubes.

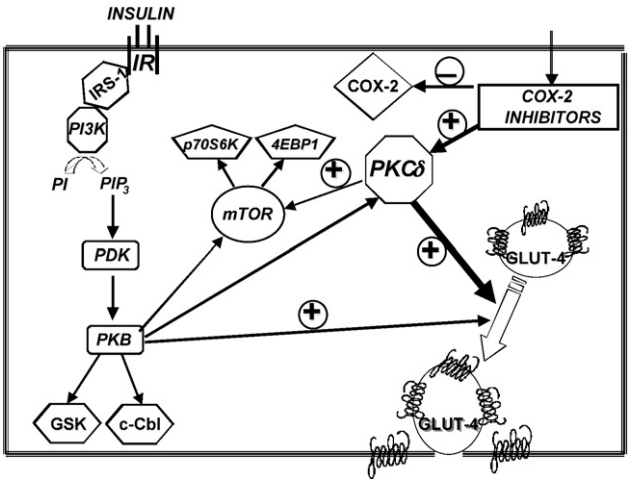

Fig. 7 – Insulin and COC-2 inhibitors augment the rate of hexose transport in L6 myotubes by two independent pathways. This models shows that COX-2 inhibitors activate PKCδ, which in turn increase the abundance of GLUT-4 in the plasma membrane of myocytes. Insulin activates its specific receptor-mediated transduction pathway, which recruits GLUT-4 containing vesicles to the plasma membrane. In addition it may also activate PKCδ that may contribute further to this interaction. The model also stresses that an activation of mTOR, either by insulin or COX-2 inhibitors, is not required for the glucose transport stimulatory effects of these agents.

functions in cells [39]. For example, tyrosine^{311} in PKCδ is a direct target for Src-dependent phosphorylation in rat cardiomyocytes [39]. It would be interesting to investigate whether the insulin receptor-Src-PKCδ pathway in myotubes, described by Rosenzwig el al. [16], entails such tyrosine^{311} phosphorylation. Conversely, the phosphorylation of tyrosine^{332} may explain the inhibitory effect of PKCδ reported by Greene et al [37]: Phosphotyrosine^{332} in PKCδ forms a docking site for the SH2 domain of Shc, which further recruits the SH2-domain-containing inositol 5′-phosphatase-1 (SHIP). The latter dephosphorylates PI-3,4,5-trisposphate and negatively regulates PKB/Akt activation [39]. There are more examples of opposing effects of PKCδ in other experimental systems: For instance, oxidative stress-induced activation of PKCδ results in its translocation to mitochondria, loss of mitochondrial transmembrane potential, release of cytochrome *c* and apoptosis of transformed cells, whereas, exposure of the same cells to fresh serum also activates of PKCδ, but triggers antiapoptotic reactions [40]. The specific pattern of COX-2 inhibitor-induced tyrosine phosphorylation of PKCδ and its subcellular localisation in relation to the upregulation of the glucose transport system in L6 myotubes remains to be investigated.

COX-2 inhibitors induced phosphorylation and reduced content of IRS-2, but not IRS-1. The activation of IRS-1 is obligatory for insulin-dependent recruitment of GLUT-4 to the plasma membrane of cells [2]. Yet, the insulin-induced IRS-2-insulin receptor complex is not believed to contribute significantly to the translocation of GLUT-4-containing vesicles to the plasma membrane. Indeed, it has been proposed that IRS-1- and IRS-2-signaling overlap incompletely and might play a broader role than just mediating insulin effects, by functioning also as proximal substrates for other signaling pathways [5,41,42]. Our data point to a COX-2 inhibitor-dependent reduction of IRS-2 protein following its hyper-phosphorylation. This could result primarily from a PKCδ-dependent activation of mTOR [22–24], because the latter induces IRS-2 degradation following the phosphorylation of selected serine residues [43–45]. Hitherto, IRS-2 depletion has not been associated with increased GLUT-4 abundance in the plasma membrane of skeletal muscle cells [5]. Yet, because IRS-2 is the prominent isoform in hepatocytes, pancreatic β-cells and kidney [5,46], it would be interesting to investigate whether COX-2 inhibitor-induced hepato- and nephrotoxic effects as well as altered β-cells function [47–49] are associated with such altered expression and function of IRS-2.

Rottlerin exerted different effects in the absence or presence of COX-2 inhibitors. In the former case, it augmented the rate of hexose uptake and GLUT-1 (but not GLUT-4) expression of the cells, possibly due to its mitochondrial uncoupling activity. Yet, rottlerin lacked such effects in the latter case, where it blocked COX-2 inhibitor-induced increase in the rate of hexose uptake. These findings suggest that the mitochondrial uncoupling activity of rottlerin requires an active COX-2 and possibly prostaglandin synthesis. Interestingly, the stimulation of prostaglandin formation is listed

among other PKCδ-dependent effects in cells [50]. Further studies are needed to elucidate the impact of such putative PKCδ-COX-2 interaction in the cytoplasm or mitochondria on energy metabolism and cellular functions.

PKCδ-induced activation of mTOR has been demonstrated in several experimental systems [22–24,50], and associated with induction of apoptosis, proliferation or differentiation, or with regulation of other cellular functions [50,51]. This leads to the hypothesis that COX-2 inhibitor-induced activation of PKCδ, followed by the activation mTOR, may contribute to the development and progression of adverse effects associated with the use selective COX-2 inhibitors in man, such as the recently reported severe cardiac complications [47–49,52,53].

Finally, the molecular interactions by which COX-2 inhibitors activate PKCδ are not clear. We have shown before [1] that some common prostaglandins fail to reverse COX-2 inhibitor-induced upregulation of glucose transport in L6 myotubes. This may indicate that a direct inhibition of COX-2 is not required for the hexose transport stimulatory effects of the inhibitors and explain the lack of effects such effects of acetylsalicylic acid and indomethacin [1]. Interestingly, preliminary experiments indicate that COX-2 inhibitors-dependent activation of PKCδ entails a 6-h lag period, which is similar to that observed in the time-course analysis of the inhibitors' effect on the rate of hexose transport. This lag period may indicate that the primary effect(s) of the inhibitors (either via direct inhibition of COX-2 or by other unrelated interactions) lays upstream to PKCδ and precedes its activation.

In summary, this study shows a key role for PKCδ in mediating the hexose uptake stimulatory effects of selective COX-2 inhibitors in L6 myotubes. This unique mechanism is not related to the classical insulin-transduction pathway or to the contraction-activated AMPKα pathway. The mechanism by which COX-2 inhibitors induce site-specific phosphorylation(s) in PKCδ, the nature of the molecular targets of activated PKCδ and their effects on GLUT-4 subcellular distribution remain to be elucidated. Of a major interest is the hypothesis that adverse effects known to be associated with the clinical use of selective COX-2 inhibitors may result from the activation of PKCδ and mTOR.

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