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ARTICLE

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Cyclooxygenase-2 (PTGS2) inhibitors augment the rate of hexose transport in L6 myotubes in an insulin- and AMPKα-independent manner

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Abstract *Aims/hypothesis:* Some cyclooxygenase-2 (COX2, also known as prostaglandin-endoperoxide synthase 2 [PTGS2]) inhibitors have been shown to increase insulin sensitivity in man or induce hypoglycaemic episodes when overconsumed or taken in combination with oral hypoglycaemic drugs. These side-effects and their impact on patients are not always recognised in routine clinical practice. We investigated whether these side-effects of COX2 (PTGS2) inhibitors result from stimulation of the glucose transport system in skeletal muscle cells. *Materials and methods:* L6 myotube cultures were used to study effects of COX2 (PTGS2) inhibitors on the glucose transport system and their relationship to PTGS2 expression, insulin action and AMP-activated protein kinase α (AMPKα) activity. *Results:* The inhibitors niflumic acid, nimesulide and rofecoxib increased the rate of hexose uptake in L6 myotubes in the absence of insulin and in a dose- and time-dependent manner. They did this by increasing the total cell content of member 4 of the solute carrier family 2 (SCLC2A4, previously known as glucose transporter 4 [GLUT4]) (but not SCLC2A1 [previously known as GLUT1]) mRNA and protein and the amount of it in the plasma membrane. AMPKα was not involved in the latter effect since the inhibitors did not activate it. In addition, none of the inhibitors modulated the rate of hexose transport in vascular endothelial and smooth muscle cells expressing PTGS2 and SCLC2A1. Prostaglandin-endoperoxide synthase 1 (also known as cyclooxygenase 1) inhibitors (acetylsalicylic acid and indomethacin) did not alter the rate of hexose uptake and SCLC2A4 subcellular distribution in L6 myotubes. *Conclusions/interpretation:* This study suggests that certain COX2 (PTGS2) inhibitors can alter glucose homeostasis in vivo by stimulating glucose uptake in skeletal muscles that express PTGS2.

Keywords Cyclooxygenase inhibitors · Diabetes · Drug interaction · Glucose transport · Hypoglycaemia · Niflumic acid · Nimesulide · Rofecoxib · Skeletal muscle

Abbreviations AICAR: 5-aminoimidazole-4-carboxamide-1-β-ribofuranosyl 5′-monophosphate · AMPKα: AMP-activated protein kinase α · COX: cyclooxygenase · dGlc: 2-deoxy-D-glucose · IKKβ: IκB kinase β · LPS: lipopolysaccharide · SCLC2A1: solute carrier family 2 (facilitated glucose transporter) member 1 · SCLC2A4: solute carrier family 2 (facilitated glucose transporter) member 4 · αMEM: α-minimal essential medium · PTGS: prostaglandin-endoperoxide synthase · VEC: vascular endothelial cells · VSMC: vascular smooth muscle cells

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Introduction

Inhibitors of prostaglandin endoperoxide synthase 2 (PTGS2, also known as cyclooxygenase 2 [COX2]) are potent anti-inflammatory drugs with reduced gastrointestinal side-effects. However, some of these drugs have serious adverse effects [1–4]. Among the less acknowledged side-effects are those on glucose homeostasis. For instance, inhibition of PTGS2 has been shown to improve insulin sensitivity in healthy subjects [5], whereas some case reports show that such inhibitors induce hypoglycaemic episodes when consumed in excess or in a combined therapy with oral hypoglycaemic drugs [6–10]. Other reports have shown that inhibition of PTGS2 prevented lipopolysaccharide (LPS)-induced impairment of glucose metabolism and insulin action in rat cardiac muscle [11].

Interestingly, a polymorphic form of the gene encoding PTGS2 has been associated with susceptibility to type 2 diabetes in Pima Indians [12]. Moreover, hyperglycaemia in itself induces PTGS2 expression in certain cells and tissues [13–15]. Yet it is not clear whether such PTGS2 induction occurs in insulin-sensitive tissues, such as skeletal muscles.

This study investigated potential effects of three COX2 (PTGS2) inhibitors, niflumic acid, nimesulide and rofecoxib, on the glucose transport system in cultured L6 myotubes. The general mechanism of PTGS2 inhibition by its selective inhibitors is complex: they interact readily and reversibly with the enzyme and induce semi-stable enzyme-inhibitor complexes. Once the drug dissociates from the complex, the recovery of the enzyme to its fully active form is slow [16]. In contrast, these compounds inhibit the constitutively expressed prostaglandin endoperoxide synthase 1 (PTGS1, also known as COX1) in a classical competitive manner, albeit with a significantly lower affinity than that required for PTGS2 inhibition [17]. Niflumic acid is a prototype COX2 (PTGS2) inhibitor that served for the development of other COX2 (PTGS2) inhibitors. It is considered a preferential inhibitor of PTGS2, but there are reports claiming equal selectivity for both PTGS1 and PTGS2 [17]. Nimesulide was one of the early generation of prescribed COX2 (PTGS2) inhibitors, but it was withdrawn from the market in some countries, or its application was restricted, because of severe renal and hepatotoxic complications [1, 18–21]. Several case reports showed that overconsumption of nimesulide or its combination with oral hypoglycaemic drugs was associated with hypoglycaemic episodes in infants and elderly individuals [6, 7, 9]. Other trials did not document similar interactions between nimesulide and oral hypoglycaemic drugs [6, 22, 23]. Rofecoxib, one of the new generation of COX2 (PTGS2) inhibitors, was, at the start of this study, one of the biggest selling COX2 (PTGS2) inhibitors of its kind. It has recently been withdrawn from markets worldwide for reassessment of its safety because of reported incidents of increased rates of cardiovascular complications [3, 24].

Materials and methods

Materials

Tissue culture media and sera were from Biological Industries (Beth–Haemek, Israel). [α-^{32}P]dCTP (111 TBq/mmol) was from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). American Radiolabeled Chemicals (St Louis, MO, USA) supplied 2-[1,2-^{3}H(N)] deoxy-D-glucose (2.22 TBq/mmol). Acetylsalicylic acid, 5-aminoimidazole-4-carboxamide-1-β-ribofuranosyl 5′-monophosphate (AICAR), 2-deoxy-D-glucose (dGlc), indomethacin, LPS (E. coli serotype 055.B), niflumic acid, nimesulide and Tri Reagent were purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA). Rofecoxib and Compound 1229 were kindly donated by Merck Research Laboratories (Rahway, NJ, USA) and Aventis Pharma (Frankfurt, Germany), respectively. Upstate Biotechnology (Lake Placid, NY, USA) supplied rabbit anti-COX2 (PTGS2) polyclonal antibody. Rabbit anti-AMPKα and anti-pT^{172}-AMPKα antibodies were from Cell Signaling Technology (Beverly, MA, USA). Enzymes, buffers and reagents for RT-PCR were purchased from Promega (Madison, WI, USA).

Animals

Male Goto–Kakizaki (GK) hyperglycaemic rats (180–200 g), from a colony originally established at Karolinska Institute (Stockholm, Sweden), were used in accordance with the guidelines of the Hebrew University of Jerusalem and our experiments were approved by the university ethics committee. The animals were kept in the Animal Facility Unit (23°C, two rats per cage, subjected to a 12-h dark/12-h light cycle) and provided with free access to standard chow and water.

Cell cultures

L6 skeletal myocytes were grown and allowed to differentiate to multinuclear myotubes (85–90% efficiency) in α-minimal essential medium (αMEM) supplemented with 2% (vol/vol) FCS, as described [25]. RAW 264.7 murine macrophages (courtesy of R. Gallily, Department of Immunology, Faculty of Medicine, The Hebrew University, Jerusalem, Israel) were grown and maintained as described [26]. Primary cultures of bovine aortic endothelial and smooth muscle cells were prepared, grown and characterised as described previously [27].

Hexose uptake assay

The rate of [^{3}H]dGlc uptake in myotubes was measured as described at 37°C [28, 29]. The effect of insulin (100 nmol/l, 20 min) was measured following a 2-h incubation period in serum-free αMEM supplemented with 0.5% (wt/vol) BSA. The inhibitors were diluted in culture medium 1:1000 from DMSO stock solutions. Uptake of dGlc was calculated on the basis of protein content per plate (L6 myotubes) or cell number (all other cells).

PCR analyses of *Sclc2a1 (Glut1)*, *Sclc2a4 (Glut4)* and *Ptgs2* mRNA

Total RNA was extracted from cell cultures using Tri Reagent according to the manufacturer's protocol. Sense and antisense primers for RT-PCR reactions of rat *Sclc2a1* and *Sclc2a4* and bovine *Ptgs2* were synthesised according to published sequences [30–32] by Sigma-Aldrich (Rehovot, Israel). Synthesis of cDNA and PCR was carried out in a PTC-100 programmable thermal controller (MJ Research, Waltman, MA, USA) as described [29–32]. The

nucleotide sequence of each PCR product was identical to the predicted sequences. Sequence analyses were performed at the DNA Analysis Unit of the Hebrew University, Jerusalem.

Preparation of cell lysates and western blot analyses

Soluble protein fractions were prepared from Triton X-100-treated cells in the presence of protease and phosphatase inhibitors, as described [29]. Protein samples (10–60 μg) were electrophoresed (6–12% SDS-PAGE). Western blot analyses of SCLC2A1 and SCLC2A4 were performed as described [29], and those for PTGS2, AMPKα and pT^{172}-AMPKα were performed according to the antibody supplier's protocol.

Cell surface biotinylation and measurement of cell surface SCLC2A1 (GLUT1) and SCLC2A4 (GLUT4)

Surface biotinylation of L6 myotubes, isolation of biotinylated proteins and western blotting of cell surface-localised glucose transporters were performed as previously described [29, 33].

Statistical analysis

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

Results

Regulation of PTGS2 (COX2) expression in L6 myotubes and in skeletal muscles

Myotubes that were exposed to 23.0 mmol/l glucose increased the expression of PTGS2 protein 3.05±0.66-fold (n=3) in comparison with the 5.5 mmol/l glucose incubation (Fig. 1a). This effect was accompanied by a nearly 2.5-fold increase in *Ptgs2* mRNA expression (Fig. 1, inset). The addition of nimesulide, niflumic acid or rofecoxib to the high-glucose culture medium for 16 h further increased PTGS2 expression by a factor of 1.43±0.07, 1.55±0.08 and 1.53±0.09, respectively, above the expression level at 23.0 mmol/l glucose. These findings agree with previous reports on hyperglycaemia-induced PTGS2 expression in other types of cells [13–15, 34]. Short-term exposure to insulin had no effect on the PTGS2 protein level in myotubes. Fig. 1b shows that an in vivo systemic inflammatory reaction following an LPS injection into diabetic GK rats caused extensive PTGS2 expression in skeletal muscles. LPS-treated RAW 264.7 murine macrophages served as a positive control in this experiment to detect LPS-dependent PTGS2 expression [35].

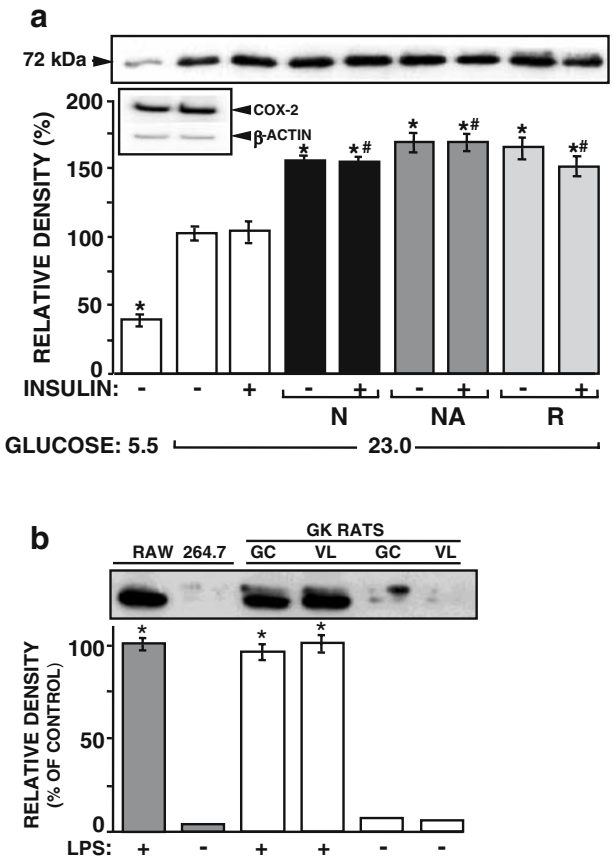


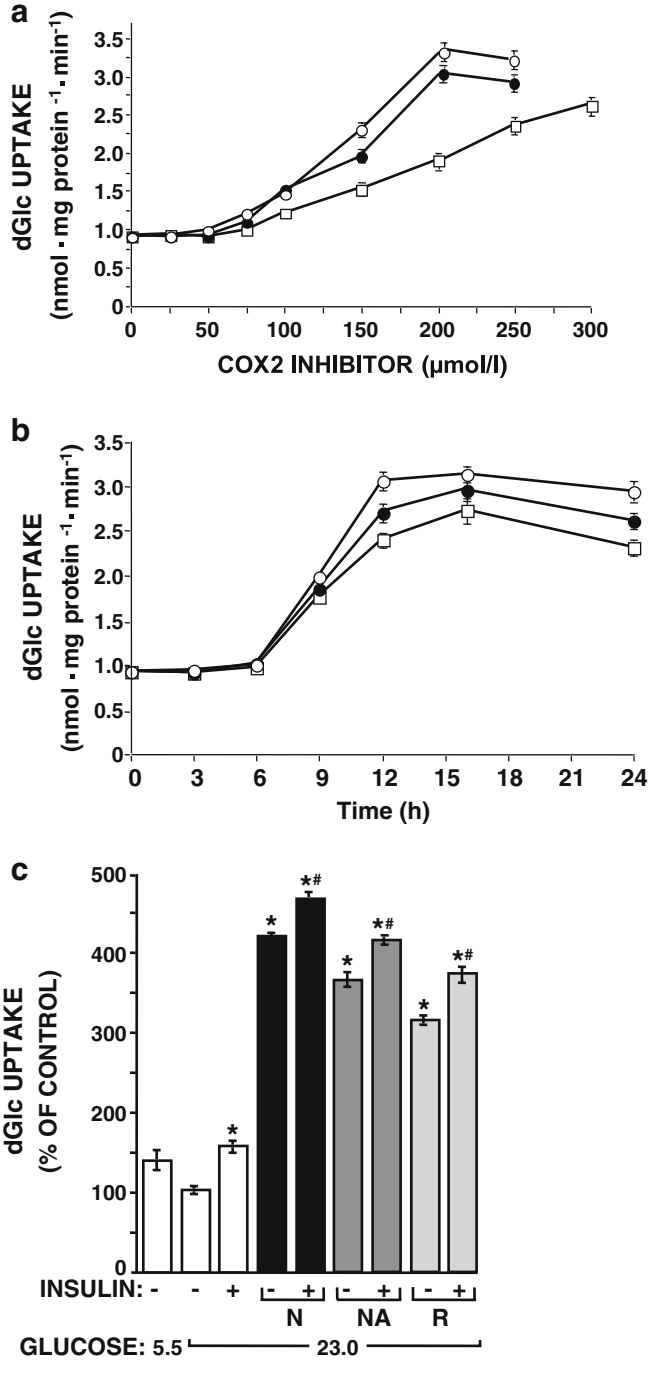
Fig. 1 **a** High glucose level and COX2 (PTGS2) inhibitors increase PTGS2 expression in L6 myotubes. Cultures that had been grown and maintained at 5.5 mmol/l glucose were exposed to 23.0 mmol/l glucose for 24 h. The cultures were then treated for 16 h with 200 μmol/l of nimesulide (*N*) or niflumic acid (*NA*) or 300 μmol/l of rofecoxib (*R*) and/or for 20 min with 100 nmol/l insulin. The panel shows a representative western blot analysis of PTGS2, plus a summary of three independent experiments. The 100% value was assigned to control myotubes incubated at 23.0 mmol/l glucose. Mean±SEM; p<0.05 for difference from the respective control L6 myotubes incubated at 5.5 mmol/l glucose or 23.0 mmol/l glucose *without or #with insulin treatment. *Inset* RT-PCR analysis of *Ptgs2* mRNA in L6 myotubes exposed to 5.5 or 23.0 mmol/l glucose. **b** Systemic inflammatory reaction increased PTGS2 expression in skeletal muscles of GK rats. Panel shows a representative western blot of PTGS2 expression in RAW 264.7 macrophages (without or with LPS treatment, 1 μg/ml, 24 h) and gluteus maximus (*GC*) and vastus lateralis (*VL*) muscles of control and LPS-treated (10 mg/kg body weight, i.p.) diabetic GK rats. The bar graph shows densitometric analysis of independent experiments. Blood glucose levels were 9.8±0.4 and 8.9±0.3 mmol/l for control and LPS-treated rats (mean±SEM, n=3). Animals were killed 24 h after the injection of LPS or the vehicle. *p<0.05 for difference from each respective control

Niflumic acid, nimesulide and rofecoxib augmented hexose uptake in PTGS2-expressing L6 myotubes

Nimesulide, niflumic acid and rofecoxib increased the rate of glucose transport in L6 myotubes nearly three-fold in a dose- and time-dependent manner: half-maximal and maximal effects were observed at 140 and 200 μmol/l of nimesulide and niflumic acid, and 170 and 300 μmol/l of rofecoxib, respectively (Fig. 2a). Maximal effects of the

three inhibitors were observed within 12–15 h (Fig. 2b). Insulin was not required for COX2 (PTGS2) inhibitor-dependent stimulation of hexose transport; in fact, the combined stimulatory effects of the inhibitors and insulin were additive (Fig. 2c). In contrast to their marked effect on L6 myotubes exposed to high glucose levels, the inhibitors increased the rate of hexose transport in L6 myotubes maintained at 5.5 mmol/l glucose approximately 1.5-fold (data not shown). Myotube viability was not affected at these concentrations of inhibitors up to a 24-h incubation period. The water solubility of these inhibitors is limited; thus, similar experiments using differentiated myotubes incubated with αMEM containing 10% (vol/vol) FCS were conducted. Half-maximal and maximal effects of niflumic acid and nimesulide were obtained at 50 and 75 μmol/l, respectively, and at 100 and 150 μmol/l for rofecoxib. The time required to reach maximal effects remained at 12–15 h. These lower effective concentrations reflect increased protein-binding kinetics of the inhibitors at the higher serum concentration. However, since L6 myotubes are routinely grown and maintained with 2% (vol/vol) FCS, we adhered to this protocol. The small increase in medium osmolarity of the 23.0 mmol/l glucose medium had no impact on the rate of dGlc uptake in L6 myotubes, which remained similar in myotubes exposed to 5.5 mmol/glucose or to 5.5 mmol/l glucose supplemented with 17.5 mmol/l L-glucose or sucrose (data not shown). We also tested the effects of these inhibitors in 3T3-L1 adipocytes, at the same effective concentrations and for the same duration of time, and found that the rate of dGlc uptake was augmented to a similar degree to that found with L6 myotubes (data not shown).

Potential effects of COX1 (PTGS1) inhibitors on the hexose transport system in L6 myotubes were also investigated. The non-competitive inhibitor acetylsalicylic acid (0.1–5.0 mmol/l; 2–24 h) and the competitive inhibitor indomethacin (0.1–1.0 mmol/l; 2–24 h) had no noticeable effect on the rate of dGlc uptake in L6 myotubes maintained at 5.5 or 23.0 mmol/l glucose (data not shown). In addition, Compound 1229, an inhibitor of IκB kinase β (IKKβ) [36], was used (0.2–1.0 μmol/l, 16 h) to determine whether IKKβ was involved in COX2 (PTGS2) inhibitor-dependent effects in a manner similar to that reported for salicylates in adipocytes [37]. This inhibitor had no apparent effect on basal or COX2 (PTGS2) inhibitor-stimulated dGlc uptake in L6 myotubes (data not shown).



◀ **Fig. 2** Dose-response and time-course analyses of COX2 (PTGS2) inhibitor-dependent stimulation of the rate of dGlc uptake in L6 myotubes. **a** L6 myotubes that had been pre-exposed to 23.0 mmol/l glucose for 24 h received increasing concentrations of nimesulide (*open circles*), niflumic acid (*filled circles*), or rofecoxib (*open squares*). The rates of dGlc uptake were measured 16 h later. **b** L6 myotubes were incubated with 200 μmol/l of nimesulide (*open circles*), niflumic acid (*filled circles*) or 300 μmol/l of rofecoxib (*open squares*) and the rates of dGlc uptake were measured at the indicated times. **c** L6 myotubes that had been preincubated with 5.5 or 23.0 mmol/l glucose for 24 h were exposed to the above-mentioned concentrations of nimesulide (*N*), niflumic acid (*NA*), or rofecoxib (*R*) for an additional 16 h and then subjected to the dGlc uptake assay. The insulin effect was measured during the last 20 min of incubation, as described under Materials and methods. The 100% value was assigned to the rate of dGlc uptake of myotubes incubated at 5.5 mmol/l glucose (0.95±0.07 nmol mg protein^{-1}/cells min^{-1}). Where not shown, error bars are smaller than symbols (mean±SEM, n=3); p<0.05 for difference from the respective *control myotubes incubated with 23.0 mmol/l glucose or $^{\#}$myotubes treated with insulin

COX2 (PTGS2) inhibitors increased total SCLC2A4 (GLUT4), but not SCLC2A1 (GLUT1), content and its plasma membrane localisation in L6 myotubes

Nimesulide, niflumic acid and rofecoxib significantly increased the abundance of SCLC2A4 (Fig. 3a), but not of SCLC2A1 (Fig. 3b), in the plasma membrane, by a factor of 2.07±0.09, 1.98±0.05 and 1.83±0.08 (mean± SEM, n=3), respectively, in comparison with untreated myotubes that were also maintained at 23.0 mmol/l glucose (p<0.05). The combined effects of the inhibitors and insulin on SCLC2A4 plasma membrane localisation were additive. Panels a and b of Fig. 3 also show that the inhibitors increased the total cell content of SCLC2A4, but not SCLC2A1, by a factor of 1.51±0.04, 1.29±0.07 and 1.23±0.07, respectively, above the control level (mean±SEM,

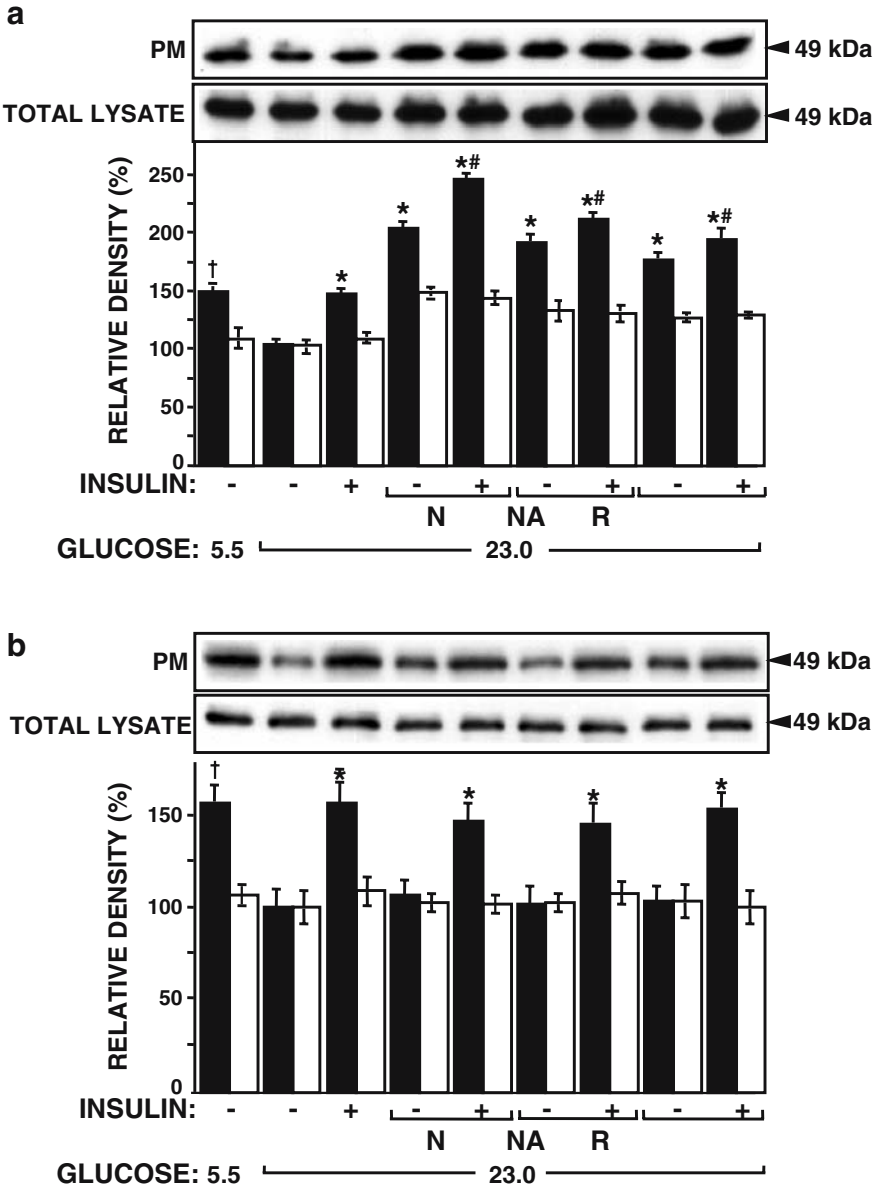
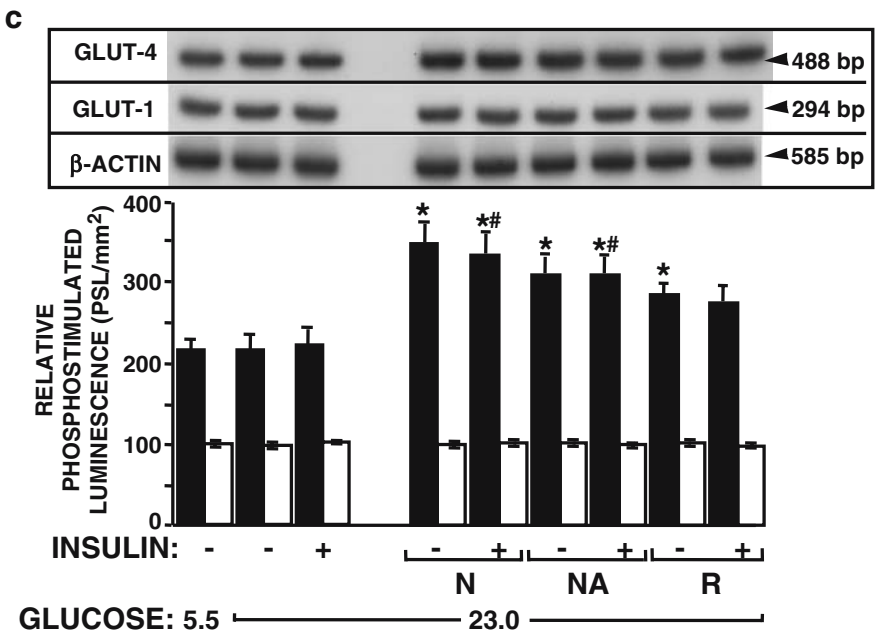


Fig. 3 COX2 (PTGS2) inhibitors increase SCLC2A4 (GLUT4), but not SCLC2A1 (GLUT1), abundance in the plasma membrane of L6 myotubes. Cultures that had been pre-exposed to 5.5 or 23.0 mmol/l glucose for 24 h were treated with 200 μmol/l of nimesulide (*N*) or niflumic acid (*NA*), or 300 μmol/l of rofecoxib (*R*) for an additional 16 h. The myotubes were then surface-biotinylated and lysed. Total cell content of glucose transporters was determined in whole cell lysates. Cell-surface-localised transporters were measured in purified biotinylated proteins. **a** Panel shows a representative SCLC2A4 western blot analysis in whole cell lysates and in cell-surface-biotinylated fractions (*PM*), plus a summary of three independent experiments (*filled bars*, plasma membrane-localised transporter; *open bars*, whole cell lysates). **b** Western blot analysis of SCLC2A1 in samples of the same cell lysates and biotinylated protein fractions. **c** RT-PCR analysis of *Sclc2a1* and *Sclc2a4* mRNA in cells treated similarly. Panel shows a representative blot, plus phosphostimulated luminescence analyses of three independent experiments. *Open bars Sclc2a1* mRNA; *filled bars Sclc2a4* mRNA. The 100% values in **a**–**c** were assigned to control cells incubated at 23.0 mmol/l glucose. Mean±SEM; p<0.05, for difference from the respective control L6 myotubes incubated at 23.0 mmol/l glucose †,*without or $^{\#}$with insulin treatment

Fig. 3 (continued)



n=3). Fig. 3c depicts a noticeable increase in *Sclc2a4* mRNA level, but not *Sclc2a1* mRNA, in myotubes treated with niflumic acid, nimesulide or rofecoxib (increased by a factor of 1.59±0.12, 1.42±0.11 and 1.31±0.09, respectively; mean ±SEM, *n*=3, $p<0.05$). Short-term incubations with insulin in the absence or presence of the inhibitors had no significant effect on *Sclc2a1* or *Sclc2a4* mRNA and protein content in L6 myotubes.

COX2 (PTGS2) inhibitors did not activate AMPKα in L6 myotubes

SCLC2A4 (GLUT4) translocation to the plasma membrane in skeletal muscle cells can also be mediated by non-insulin-dependent stimuli: exercise, muscle contraction and hypoxia activate AMPKα, which augments SCLC2A4 translocation to the plasma membrane in skeletal muscles. Also, the synthetic AMP analogue AICAR activates AMPKα, as does a short-term hyperosmolar shock [38–40]. These stimuli induce specific threonine^{172}-phosphorylation in AMPKα, which is obligatory for its activation [38, 40]. Fig. 4 shows such specific pT^{172}-AMPKα phosphorylation following an AICAR treatment or a short-term exposure of myotubes to 500 mmol/l D-sorbitol. As expected, insulin, but also COX2 (PTGS2) inhibitors, had no such effect on AMPKα.

COX2 (PTGS2) inhibitors failed to alter the rate of hexose transport in PTGS2–expressing vascular cells

Bovine aortic endothelial cells (VEC) and smooth muscle cells (VSMC), which predominantly express SCLC2A1 (GLUT1) [30], were used to investigate the potential effects of COX2 (PTGS2) inhibitors on their hexose transport capacity. Fig. 5a shows a lack of *Ptgs2* mRNA and protein expression in VEC that were maintained at 5.5 or 23.0 mmol/l glucose. However, PTGS2 expression was markedly induced in these cells following LPS treatment (1.0 μg/ml, 24 h). VSMC that were incubated at 23.0 mmol/l glucose expressed more *Ptgs2* mRNA and

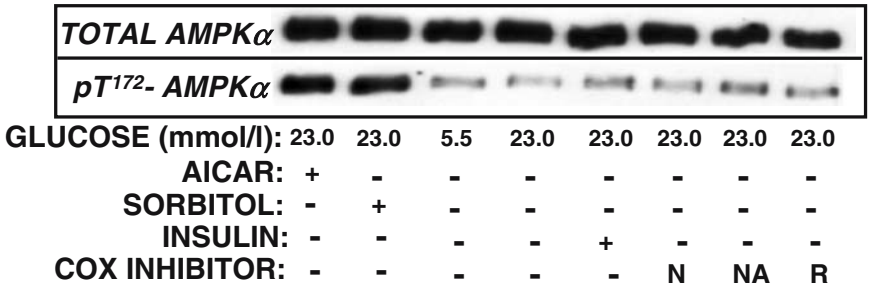


Fig. 4 COX2 (PTGS2) inhibitors do not induce tyrosine^{172}-phosphorylation in AMPKα. Lysates were prepared from L6 myotubes that had been treated with nimesulide (*N*), niflumic acid (*NA*) or rofecoxib (*R*) without or with insulin, as described in the legend to Fig. 3. Myotubes were also treated with 2 mmol/l AICAR for 1 h or 500 mmol/l D-sorbitol for 30 min. Cell lysates were then used for western blot analyses with antibodies against AMPKα (62 kDa) or pT^{172}-AMPKα

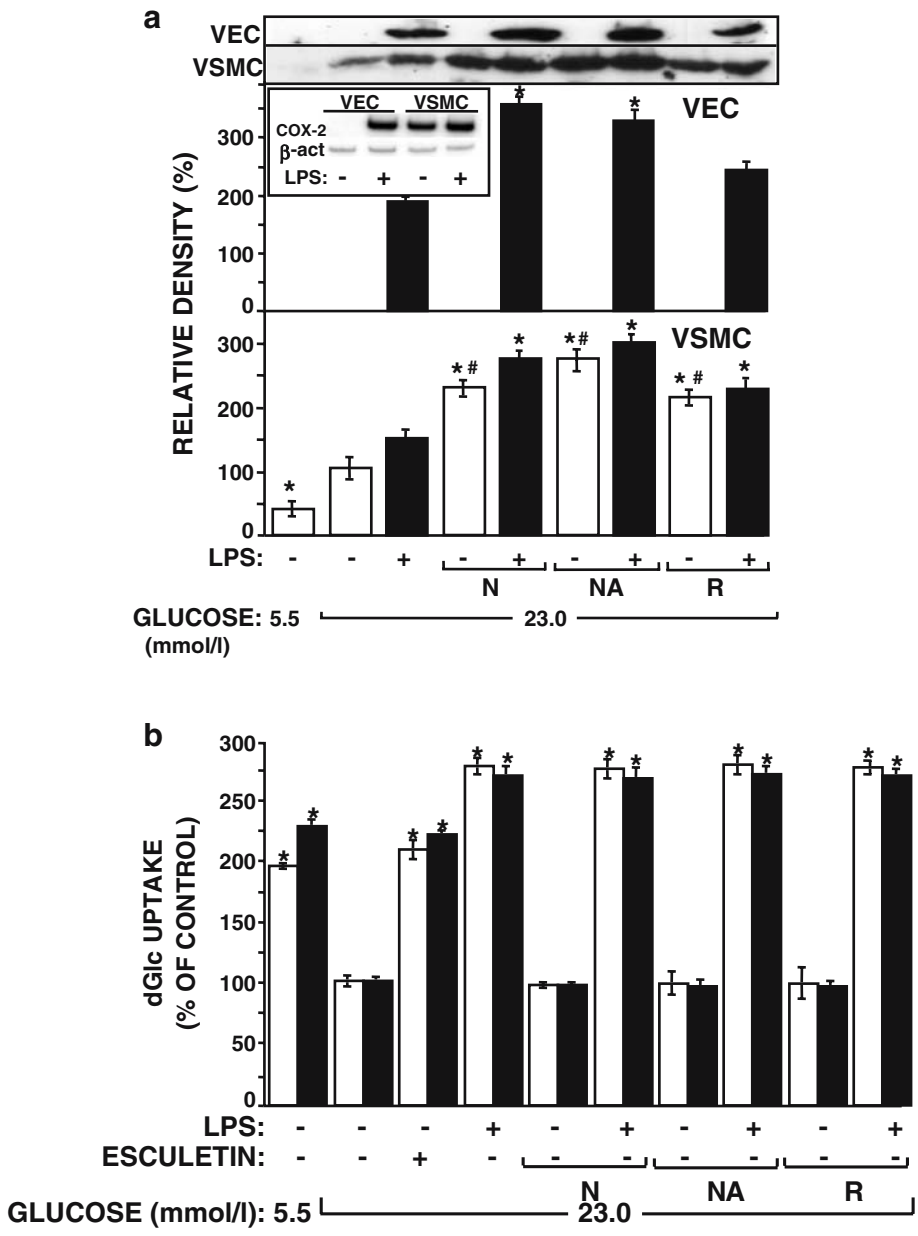


Fig. 5 Effects of high glucose level, LPS and COX2 (PTGS2) inhibitors on PTGS2 expression and the rate of dGlc uptake in vascular cells. VEC and VSMC that had been grown and maintained at 5.5 mmol/l glucose were incubated with 23.0 mmol/l glucose for 48 h. Some cultures were treated with 1.0 μg/ml LPS during the last 24 h of incubation. All cultures were then washed and received fresh medium supplemented with 23.0 mmol/l glucose, without or with 100 μmol/l of esculetin, 200 μmol/l of nimesulide or niflumic acid, or 300 μmol/l of rofecoxib and incubated further for 16 h. **a** Representative western blot analysis of PTGS2 in whole VEC and VSMC lysates (*open bars*, control cells; *filled bars*, LPS-treated cells) and a summary of densitometric analyses of three independent experiments. **b** Rates of dGlc uptake in VEC (*open bars*) and VSMC (*filled bars*) that were treated similarly. The 100% dGlc uptake values were assigned to VEC and VSMC that were incubated at 23.0 mmol/l glucose with no other additions (42±6 and 106±11 pmol dGlc 10^{-6} cells min^{-1}, respectively; mean±SEM, $n=3$); $p<0.05$ for difference from the respective control L6 myotubes incubated at 23.0 mmol/l glucose *without or #with LPS treatments

protein than cells at 5.5 mmol/l glucose; LPS treatment further augmented this expression. Fig. 5b shows that none of the COX2 (PTGS2) inhibitors used altered the rates of dGlc uptake in vascular cells before or after LPS challenge. We have shown before that the inhibition of arachidonate 12-lipoxygenase by the inhibitor esculetin augments the rate of hexose transport in these vascular cells [29]. VEC or VSMC that were exposed to esculetin augmented the rate of dGlc uptake regardless of the presence of COX2 (PTGS2) inhibitors or PTGS2 induction by LPS.

Discussion

This study shows that COX2 (PTGS2) inhibitors augment the rate of hexose transport in L6 myotubes in a dose- and time-dependent manner, and that this effect is more prominent in myotubes exposed to high glucose levels, in which PTGS2 expression is increased, than in myotubes exposed to 5.5 mmol/l glucose. The major effect of the inhibitors is the recruitment of SCLC2A4 (GLUT4), but not SCLC2A1 (GLUT1), to the plasma membrane. A very low level of SCLC2A3 (GLUT3) expression in the L6 cell line used in this study precluded reliable determinations of the total and plasma membrane abundance of this transporter. The failure of COX2 (PTGS2) inhibitors to alter SCLC2A1 abundance in the plasma membrane of L6 myocytes or to modulate the rate of hexose transport in PTGS2-expressing vascular endothelial and smooth muscle cells indicates that these inhibitors affect predominantly SCLC2A4-mediated glucose transport. The observation that COX2 (PTGS2) inhibitors themselves increased the level of PTGS2 expression in skeletal muscle cells is interesting. It is unclear whether such induction of PTGS2 occurs in vivo.

The present data show that the level of COX2 (PTGS2) inhibitor-dependent stimulation of hexose uptake (approximately three-fold; Fig. 2) was higher than the corresponding relative recruitment of SCLC2A4 to the plasma membrane (approximately two-fold; Fig. 3a). Thus, it remains to be investigated whether COX2 (PTGS2) inhibitors increase the intrinsic activity of GLUT4 in the plasma membrane of myotubes, in addition to their augmenting effect on GLUT4 abundance in this compartment.

The molecular interactions of COX2 (PTGS2) inhibitor-induced augmentation of hexose uptake are not fully understood. The present results show that the effects of insulin on the glucose transport system and SCLC2A4 subcellular distribution on the one hand and of the inhibitors on the other were not additive. In fact, insulin further stimulated the rate of hexose uptake as well as SCL C2A4 translocation to the plasma membrane in myotubes that were already exposed to maximal effective concentrations of COX2 (PTGS2) inhibitors (Figs. 2c and 3a). Therefore, it is unlikely that the inhibitors utilise the insulin transduction mechanism to exert their effects. Indeed, preliminary experiments indicated that COX2 (PTGS2) inhibitors failed to mimic insulin and induce targeted tyrosine and serine phosphorylations in IRS-1 and protein kinase B/Akt (data not shown). Moreover, the alternative AMPKα-dependent pathway, which stimulates hexose transport in skeletal muscles by recruiting SCLC2A4 to the plasma membrane upon reduction in cellular energy charge and following muscle contraction [38–40], was not activated by any of the COX2 (PTGS2) inhibitors tested. More investigations, based upon preliminary observations in our laboratory linking the effects of COX2 (PTGS2) inhibitors to the activation of protein kinase Cδ [41], may ascertain the molecular mechanism by which COX2 (PTGS2) inhibitors stimulate glucose transport in SCL C2A4 expressing cells and tissues.

It is not known whether COX2 (PTGS2) inhibitor-dependent stimulation of glucose transport results from direct inhibition of PTGS2. The findings on the stronger effects of the inhibitors in L6 myotubes expressing high levels of PTGS2 under hyperglycaemic conditions seem to support this notion. The specific PTGS2 product involved in this function is still unknown. Long and Pekala have suggested that SCLC2A4 expression in 3T3-L1 adipocytes is suppressed by prostaglandin (PG) $E_{2\alpha}$ [42], while Chiou and Fong showed a $PGF_{2\alpha}$-dependent increased expression of SCLC2A1 in these cells [43]. In the present study, $PGE_{2\alpha}$, $PGF_{2\alpha}$ and $PGA_{2\alpha}$ failed to modulate the rate of glucose transport when added alone or together with COX2 (PTGS2) inhibitors to L6 myotubes (data not shown). It is possible that these molecules were rapidly oxidised during the relatively long incubation period, failing to reach minimal effective concentrations in cells.

Acetylsalicylic acid has been shown to reverse insulin resistance in 3T3-L1 adipocytes by inhibiting IKKβ [37]. In the present study both acetylsalicylic acid and indomethacin failed to modulate glucose transport in myotubes. Furthermore, in order to exclude an IKKβ-dependent interaction in myotubes we tested Compound 1229, an inhibitor of IKKβ, alone or in combination with COX2 (PTGS2) inhibitors, and found no evidence for such interactions.

The present findings seem highly relevant to case reports on COX2 (PTGS2) inhibitor-induced hypoglycaemic episodes in man. We suggest that vulnerable diabetic patients are those who receive, in addition to conventional oral hypoglycaemic drugs, COX2 (PTGS2) inhibitors to relieve symptoms of chronic or acute inflammatory processes. While some individuals from this group could benefit from COX2 (PTGS2) inhibitor therapy through having their blood glucose level reduced towards the normoglycaemic range, other susceptible patients might experience an excessive reduction in blood glucose level towards the hypoglycaemic range. We suspect that the individuals at risk are those in whom PTGS2 expression is increased excessively in skeletal muscles in the presence of inflammatory mediators. Our findings on LPS-induced expression of PTGS2 in skeletal muscles of diabetic GK rats support this hypothesis (Fig. 1b).

In conclusion, this study shows that nimesulide, niflumic acid and rofecoxib increase the expression of SCLC2A4 and its plasma membrane localisation as well as the rate of hexose uptake in L6 myotubes in a dose-, time- and non-insulin-dependent manner. It is not yet established whether such stimulatory effects are shared with all other available COX2 (PTGS2) inhibitors. However, such possible interactions of these inhibitors in susceptible individuals make it necessary for physicians to be aware of this problem and to carefully monitor patients in order to optimise drug treatment and avoid adverse interactions.

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