International Journal of Pharmaceutics 428 (2012) 25–32

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Regulation of human monocarboxylate transporter 4 in skeletal muscle cells: The role of protein kinase C (PKC)

Katsuya Narumi[a,b,1], Masaki Kobayashi[a,1], Sho Otake[a], Ayako Furugen[a], Natsuko Takahashi[c], Jiro Ogura[a], Shirou Itagaki[a,2], Takeshi Hirano[a,3], Hiroaki Yamaguchi[a], Ken Iseki[a,b,*]

[a] Laboratory of Clinical Pharmaceutics and Therapeutics, Division of Pharmasciences, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12-jo, Nishi-6-chome, Kita-ku, Sapporo 060-0812, Japan
[b] Department of Pharmacy, Hokkaido University Hospital, Sapporo 060-8648, Japan
[c] Graduate School of Medicine, Hokkaido University, Sapporo 060-8638, Japan

ARTICLE INFO

Article history:
Received 3 October 2011
Received in revised form 30 January 2012
Accepted 10 February 2012
Available online 9 March 2012

Keywords:
Monocarboxylate transporter 4
Skeletal muscle
Protein kinase C
Hypoxia-inducible factor 1α

ABSTRACT

In the present study, to clarify the role of protein kinase C (PKC) in the regulation of monocarboxylate transporter 4 (MCT4) expression, we examined the regulation mechanism of MCT4 expression in human rhabdomyosarcoma (RD) cells, an in vitro skeletal muscle model. Exposure of RD cells to PMA, a PKC activator, for 24 h resulted in a two-fold increase in the amount of lactic acid in the growth medium. In parallel to an increase in lactic acid release from RD cells, the level of MCT4 mRNA and protein were also significantly increased in RD cells. A PKC inhibitory study indicated that PMA-induced stimulation of MCT4 expression can be mediated through a novel PKC isoform, especially PKCδ. Moreover, rottlerin, a selective PKCδ inhibitor, decreased PMA-induced MCT4 promoter activity. Deletion and mutational analysis suggested that the potential hypoxia-response elements (HREs) played a major role in the observed modulation of MCT4 expression by PMA. Furthermore, we found that small interfering RNA (siRNA)-mediated knockdown of hypoxia-inducible factor 1α (HIF-1α) significantly inhibited PMA-induced MCT4 promoter activity. Our results show that the effects of PMA on MCT4 expression are mediated through an indirect pathway partially involving PKCδ and HIF-1α transcription factor.

1. Introduction

Skeletal muscle, which plays a crucial role in glycolytic metabolism for maintenance of homeostasis, is the main producer of lactic acid in the body. The intracellular content of lactic acid is a product of both production, mediated by increases in glycolysis, and removal, mediated by transport, either out of the cell or into the mitochondria. If the efflux of lactic acid from the cell does not keep pace with production, concentrations of lactic acid increase and induce cytosolic acidification (Jeong et al., 2001). Transport of lactic acid across the plasma membrane of almost all cells is catalyzed by proton-linked monocarboxylate transporters (MCTs). The MCT family now comprises 14 members, of which the first four (MCT1–MCT4) subtypes have been demonstrated experimentally to catalyze the proton-linked transport of metabolically important monocarboxylates such as L-lactic acid (Halestrap and Meredith, 2004). Distinct kinetic properties for lactic acid and pyruvate have been shown for MCT1 and 4 (Broer et al., 1998; Dimmer et al., 2000), all of which are expressed in skeletal muscle (Bonen et al., 2000a, 2006; Benton et al., 2004). MCT1 has been found in all fiber types in muscle tissue, but it is more abundant in muscles that have a high percentage of oxidative cells than in glycolytic muscles and thus has been suggested to play a major role in influx of lactic acid for oxidation (Pilegaard et al., 1999a; Juel and Halestrap, 1999). The other major MCT isoform found in muscles, especially in white muscle fibers, is MCT4 (Wilson et al., 1998), which is thought to play a primary role in the efflux of L-lactic acid from muscle fibers, and its expression has been shown to be associated with indices of glycolytic capacities (Bonen et al., 2000b). It has been shown that lactic acid transport and MCT protein expression can be altered in relation to the metabolic demands placed on muscle by contractile activity. For example, transport of lactic acid can be increased when muscle activity is chronically increased, either by training (McDermott and Bonen, 1993; Pilegaard et al., 1993, 1999b; Bonen et al., 1998; Dubouchaud et al., 2000) or by chronic

* Corresponding author at: Laboratory of Clinical Pharmaceutics & Therapeutics, Division of Pharmasciences, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12-jo, Nishi-6-chome, Kita-ku, Sapporo 060-0812, Japan.
Tel.: +81 11 706 3770; fax: +81 11 706 3770.
E-mail address: ken-i@pharm.hokudai.ac.jp (K. Iseki).
[1] These authors contributed equally as first author.
[2] Present address: Department of Pharmacy, Hirosaki University School of Medicine & Hospital, Hirosaki, Japan.
[3] Present address: Department of Pharmacy, Kobe University Hospital, Kobe, Japan.

0378-5173/$ – see front matter © 2012 Published by Elsevier B.V.
doi:10.1016/j.ijpharm.2012.02.021

electrical stimulation of rat hindlimb muscles (Bonen et al., 2000b; McCullagh et al., 1996, 1997). MCT1 has been thoroughly studied (Cuff and Shirazi-Beechey, 2002; Smith and Drewes, 2006; Benton et al., 2008; Saksena et al., 2009), but there has been very little investigation of the mechanisms controlling MCT4 expression.

Protein kinase C (PKC) plays important roles in intracellular signaling involved in many cellular responses (Dempsey et al., 2000). The PKC family comprises 11 isoforms that are classified into 3 subfamilies, classical PKC (cPKC), novel PKC (nPKC) and atypical PKC (aPKC) (Newton, 2003), based on their structure and allosteric requirements. The cPKC isoforms are regulated by secondary messengers, both calcium and phorbol esters or endogenously generated diacylglycerol (DAG). The nPKC isoforms are also activated by DAG but are insensitive to calcium, and the aPKC isoforms are independent of calcium and do not respond to phorbol esters. These PKC isoforms have different patterns of tissue expression and differentially regulate various cellular processes, including proliferation and differentiation. Skeletal muscle contraction increases intracellular calcium and has been demonstrated to increase DAG following in situ contraction in rat muscle (Cleland et al., 1989). PKC activation occurs during contractions of rat skeletal muscle (Richter et al., 1987). Moreover, there is evidence that some PKC isoforms have a role in exercise-mediated glucose transport (Cleland et al., 1989). Given that muscle activity exerts a strong influence on the expression of MCT4, it is conceivable that PKC could affect expression of MCT4 in human skeletal muscle.

Previous studies in our laboratory have demonstrated that the RD cell line can function as a suitable model to study L-lactic acid transport and have suggested that L-lactic acid efflux is mediated by MCT4 in RD cells (Kobayashi et al., 2005). In this study, we investigated the role of PKC in the regulation of MCT4 expression utilizing RD cells as an in vitro skeletal muscle model.

2. Materials and methods

2.1. Chemicals

L-Lactic acid sodium salt was purchased from ICN Biomedicals Inc (Aurora, OH). Bisindolylmaleimide (BIM) and Gö6976 were obtained from Funakoshi (Tokyo, Japan) and LC Laboratories (Woburn, MA), respectively. Phorbol 12-myristate 13-acetate (PMA) and all other chemicals were obtained from SIGMA (St. Louis, MO). These agents were dissolved in DMSO and stored at −20 °C. They were added to the incubation medium just before use.

2.2. Cell culture

Experiments were carried out using cultured human rhabdomyosarcoma cells (RD cells). RD cells of the spindle-cell type were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). RD cells, prototypic embryonal rhabdomyosarcoma cells, are tumor cells of skeletal muscle origin affecting children and young adults, and they express a number of muscle-specific proteins (Knudsen et al., 1998). The RD cells were maintained in plastic culture flasks (Corning Incorporated, Corning) as described previously (Kobayashi et al., 2005), and the cells were kept in Dulbecco's modified Eagle's medium (SIGMA) with 10% fetal bovine serum (ICN Biomedicals, Inc.) and 1% penicillin–streptomycin, respectively, at 37 °C under 5% CO_2.

2.3. Determination of lactic acid content

RD cells were seeded into 24-well plates and cultured in growth media to 80% confluency and then the cells were treated with DMSO or PMA in culture medium for 6, 12, or 24 h. Lactic acid content in the supernatant was measured spectrophotometrically using a Lactate Reagent (Trinity Biotech USA, St. Louis, MO) as per the manufacturer's protocol.

2.4. Quantitative real-time PCR

Quantitative real-time PCR was performed using an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) with 2× SYBR Green PCR Master Mix (Applied Biosystems) as per the manufacturer's protocol. PCR was performed using human MCT4-specific primers through 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; using human HIF-1α-specific primers through 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min; or using human 18S-specific primers after pre-incubation at 95 °C for 15 min. The primers specific to human MCT4, HIF-1α and 18S were designed on the basis of sequences in the GenBank™ database (accession no.: NM_004207, NM_001530 and NR_003286, respectively). The sequences of the specific primers were as follows: the sense sequence was 5′-ATT GGC CTG GTG CTG CTG ATG-3′ and the antisense sequence was 5′-CGA GTC TGC AGG AGG CTT GTG-3′ for human MCT4, the sense sequence was 5′-GAA AGC GCA AGT CCT CAA AG-3′ and the antisense sequence was 5′-TGG GTA GGA GAT GGA GAT GC-3′ for human HIF-1α and the sense sequence was 5′-CGG CTA CCA CAT CCA AGG AA-3′ and the antisense sequence was 5′-GCT GGA ATT ACC GCG GCT-3′ for human 18S. The PCR products were normalized to amplified 18S, which was the internal reference gene (housekeeping gene). Standard curves were prepared for each target and housekeeping gene. A standard curve was established between the threshold cycles (Ct) and the $\log_{10}$ (copy numbers) by using Applied Biosystems sequence detection system software, version 1.9.1. The software calculates the relative amount of the target gene and the housekeeping gene based on the Ct.

2.5. Western blot analysis

Western blot analysis was performed as described previously (Kobayashi et al., 2006). Total protein extracts were prepared from RD cells. The cells were scraped and centrifuged at 1300 × *g* for 1 min at 4 °C. The pellet was suspended in 1 mL of PBS and centrifuged at 1300 × *g* for 1 min at 4 °C. The resulting pellet was suspended in a lysis buffer containing 1.0% Triton X-100, 0.1% SDS and 4.5 M urea. The suspension was allowed to stand for 5 min and was sonicated for 15 min at 4 °C. The suspension was then centrifuged at 12,000 × *g* for 15 min at 4 °C, and the protein concentration in the clear supernatant was determined by the method of Lowry et al. (Lowry et al., 1951). The samples were denatured at 100 °C for 3 min in a loading buffer containing 50 mM Tris–HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% BPB and 3.6 M urea and separated on 4.5% stacking and 10% SDS polyacrylamide gels. Proteins were transferred electrophoretically onto nitrocellulose membranes at 15 V for 90 min. The membranes were blocked with PBS containing 0.05% Tween 20 (PBS/T) and 10% non-fat dry milk for 24 h at room temperature. After being washed with PBS/T, the membranes were incubated with rabbit anti-MCT4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (diluted 1:200) or mouse anti-actin monoclonal antibody (Chemicon, Temecula, CA) (diluted 1:500) for 24 h at room temperature and washed three times with PBS/T for 10 min each time. The membranes were subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:4000 and washed three times with PBS/T for 10 min each time. The bands were visualized by enhanced chemiluminescence

according to the instructions of the manufacturer (Amersham, Buckinghamshire, UK).

2.6. Reporter plasmid construction

The human MCT4 promoter (−2093/+249) was amplified by PCR from human genomic DNA and then subcloned into a pGL3-basic vector (Promega, Madison, WI) that contains a promoterless luciferase reporter gene. Four 5′-deletion fragments (−1499/+249, −936/+249, −505/+249, and −23/+249) were generated from −2093/+249 by the PCR amplification method and subcloned into a pGL3-basic vector. Five different forward primers contained an internal site for Xho I restriction enzyme and their sequences are primer-1, 5′-CCC CTC GAG GCC ATC ACA TCA CTG TGA CC-3′; primer-2, 5′-ACC CTC GAG CTG CTC TCC CTC CTC AGA AA-3′; primer-3, 5′-GGG CTC GAG AGG GGC CAG TTA AAG CAT TC-3′; primer-4, 5′-CGG CTC GAG AGA GGA AGC GGA GGT CTG A-3′; and primer-5, 5′-TGC CTC GAG AGC GAC CCG AGC ACT TAA A-3′. In all cases, the sequence of the reverse primer contained a site for Hind III enzyme and the sequence is 5′-CGC AAG CTT TCG AGA AGG CTA GTC CAT GC-3′. The vectors of NF-κB mut, HRE mutA and HRE mutA–B were generated from −2093/+249 by megaprimer PCR methods using forward and reverse primers for the MCT4 promoter and primers with a sense sequence of 5′-GGA GGC GAG GCC GTA ACG ACC CAG TCC CGA G-3′ for NF-κB mutation, a sense sequence of 5′-GCC AGG CGG TGA CAT ACG CGG GGG ACG TG-3′ for HRE mutation A and a sense sequence of 5′-CAT ACG CGG GGG ACA TAC CGC GCC AGC GAC C-3′ for HRE mutation A–B. All PCR products and deletion constructs for reporter assays were sequenced using an ABI PRISM 3100 Genetic Analyzer with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions.

2.7. Transient transfection and luciferase assay

RD cells (1.0×10^5 cells/well) were seeded into 24-well plates and transfected while still in suspension with one of the MCT4 promoter-luciferase constructs using Lipofectin reagent (Invitrogen, Carlsbad, CA). Each well was transfected with 400 ng of pGL3-MCT4 promoter construct encoding a modified firefly luciferase gene (or empty pGL3 vector). For HIF-1α knockdown analyses, the HIF-1α small interfering RNA (siRNA)-transfected cells (as described below) were co-transfected with the luciferase reporter plasmid. After transfection, cells were transferred to a fresh medium supplemented with PMA or DMSO (control). At 48 h after transfection, luciferase activity was measured by a luminometer according to the manufacturer's instructions using an assay kit from Promega. Relative light unit (RLU) values obtained from the assay were normalized to the amount of protein used as determined by the Lowry method (Lowry et al., 1951) and luciferase activity was expressed as RLU/mg protein.

2.8. Knockdown of HIF-1α using small interfering RNA

A Silencer® Validated siRNA targeted to MCT4 and HIF-1α gene and nontargeting siRNA as a Silencer® Negative control no. 1 siRNA were purchased from Ambion (Austin, TX). Delivery of siRNAs into RD cells was performed by reverse transfection methods as per the manufacturer's protocol. Two point five microliters of nontargeting siRNA or HIF-1α siRNA (2 μM) and 97.5 μL of OPTI-MEM® I Reduced Serum Medium (GIBCO, Grand Island, NY) were mixed in 24-well plastic plates and incubated at room temperature for 10 min after addition of 1 μL of Lipofectamine™ RNAiMAX (Invitrogen). Then 400 μL of suspended RD cells (1.0×10^5 cells/mL) in growth medium without antibiotics was added. After siRNA transfection (24 h), the cells were used for reporter gene assays as described above. Following siRNA transfection, the medium was replaced with DMEM containing 10% FBS for an additional 48 h and then the cells were analyzed by quantitative real-time PCR analysis (as described above) to determine the efficiency of knockdown.

2.9. Data analysis

Student's *t*-test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was evaluated using ANOVA followed by the Tukey–Krammer test. Statistical significance was defined as $P < 0.05$.

3. Results and discussion

3.1. Effect of PMA on lactic acid release and expression of MCT4 in RD cells

Firstly, we investigated the effect of PMA, a PKC activator, on lactic acid release from RD cells. Exposure of RD cells to PMA for 24 h resulted in a two-fold increase in the amount of lactic acid in the medium compared with that in the case of control cells (Fig. 1A). Next, we determined whether expression of MCT4, a key monocarboxylate transporter that mediate the efflux of lactic acid from cells, is modulated by PMA. Fig. 1B shows that the level of MCT4 mRNA exhibited a time-dependent response to PMA. MCT4 mRNA level was significantly increased from 12 to 24 h. The level of MCT4 mRNA was increased by approximately two-fold compared with the control (Fig. 1B). On the other hand, the PMA-induced lactic acid release from RD cells was significantly inhibited by MCT4 siRNA (Supplemental Fig. 1). MCT4 protein levels were measured by Western blotting in whole cell lysates of RD cells exposed to PMA. A significant increase in MCT4 protein level was seen when the cells were incubated with PMA for 24 h but not when cells were incubated with PMA for 6 or 12 h (Fig. 1C). Parallel to the increase in lactic acid release from RD cells in response to PMA (Fig. 1A), 24-h incubation with 0.1 μM PMA significantly increased the MCT4 protein level as well as the mRNA level. Several studies have demonstrated that long-term PMA treatment increased glucose consumption and lactic acid production (Denis-Pouxviel et al., 1990). Considering MCT4 mediates lactic acid efflux from glycolytic tissues, it is possible that its expression increases in response to increase in glycolytic rate in order to enable export of the increased quantities of lactic acid.

Supplementary material related to this article found, in the online version, at doi:10.1016/j.ijpharm.2012.02.021.

3.2. Effects of specific PKC inhibitors on PMA-induced stimulation of lactic acid release and MCT4 expression in RD cells

To investigate the role of PKC in the lactic acid release and expression of MCT4, the effects of various PKC inhibitors on PMA-induced lactic acid release and MCT4 protein levels were examined. Fig. 2 A and B shows that BIM, a specific PKC inhibitor, at 1 μM blocked the PMA-induced lactic acid release and MCT4 protein level, indicating that the effect of PMA could be mediated by stimulation of PKC. Previous studies have shown that BIM at a concentration of 1 μM is sufficient to inhibit most of the PKC isoforms (Martiny-Baron et al., 1993). Given that PMA is a DAG mimetic, it seems reasonable to assume that either cPKC or nPKC isoforms play a role in this effect. Other specific PKC isoform inhibitors including Gö6976 and rottlerin (ROT) were also used. In the presence of Gö6976, an inhibitor of cPKC, there were no significant changes in PMA-induced MCT4 protein expression (Fig. 2C). On the other hand, ROT, a selective PKCδ inhibitor, abolished the stimulatory effects of PMA (Fig. 2D). While these results do not support a role for cPKCs in

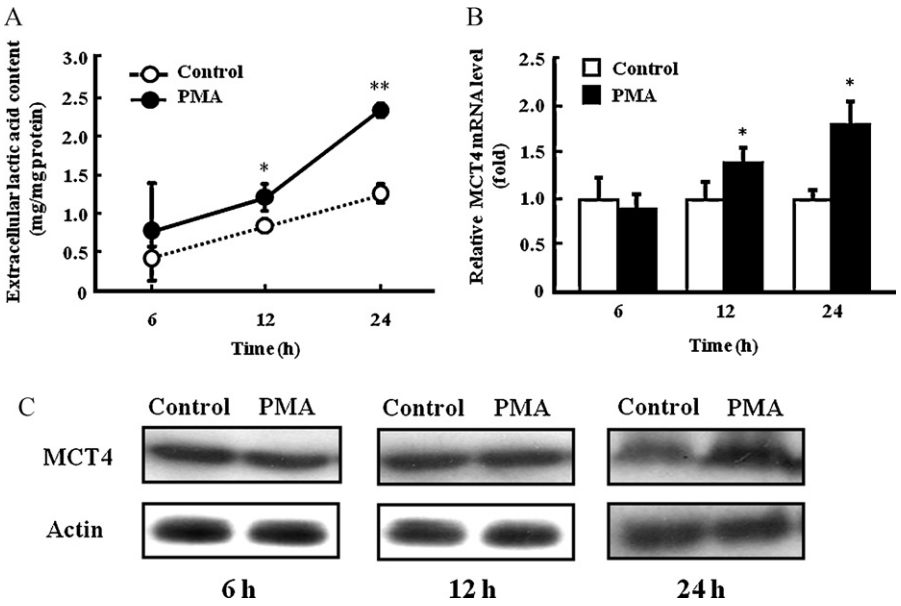

Fig. 1. Changes in lactic acid contents in culture medium (A), MCT4 mRNA (B) and protein (C) levels after treatment of RD cells with PMA. (A) RD cells were incubated in the absence or presence of 0.1 μM PMA for variable periods time (6, 12 and 24 h). Lactic acid was determined in the condition media at the indicated times. Each point represents the mean with S.D. of 3 determinations. Untreated (control) and PMA-treated cells are indicated by a white symbol and a black symbol, respectively. *, **; significantly different from control at $P < 0.05$, $P < 0.01$. (B) RD cells were exposed to PMA (0.1 μM) for 6, 12 and 24 h. MCT4 mRNA levels were determined using quantitative real-time reverse transcription-PCR. Each column represents the mean with S.D. of 6 determinations. *; significantly different from control at $P < 0.01$. (C) RD cells were exposed to PMA (0.1 μM) for 6, 12 and 24 h. Whole cell extracts were prepared from the cells and resolved using SDS-PAGE. Western blotting was carried out with antibodies to MCT4 or β-actin. Two separate experiments gave similar results.

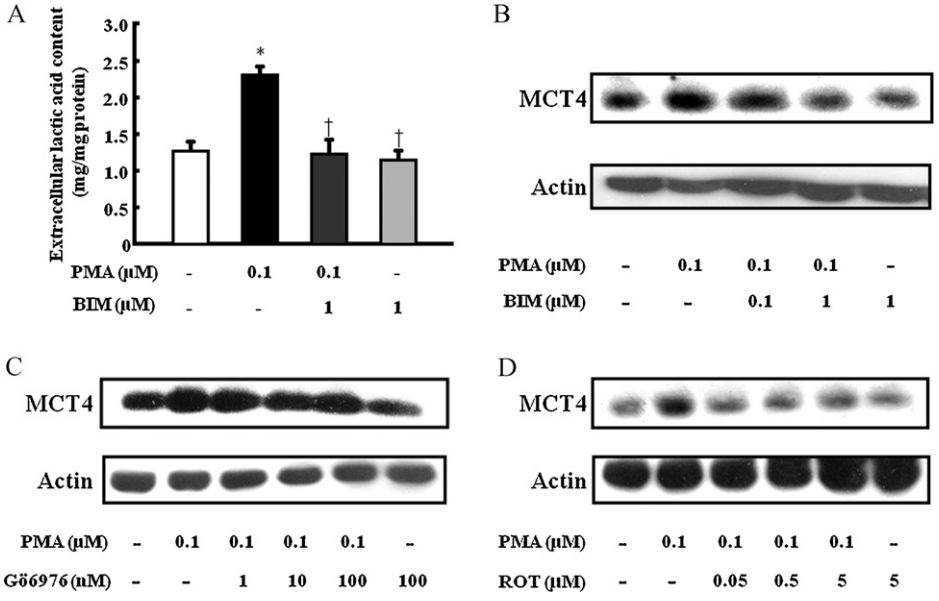

Fig. 2. Effects of PKC inhibitors on PMA-induced lactic acid secretion (A) and MCT4 protein expression (B–D) in RD cells. (A) RD cells were exposed PMA in the absence or presence of bisindolylmaleimide (BIM) for 24 h. Each column represents the mean with S.D. of 3 determinations. *; significantly different from the control at $P < 0.01$. †; significantly different from PMA alone at $P < 0.01$. (B–D) RD cells were exposed to PMA (0.1 μM) in the absence or presence of BIM, Gö6976 and rottlerin (ROT) for 24 h. Western blotting was carried out with antibodies to MCT4 or β-actin as described above. Two separate experiments gave similar results.

the regulation of MCT4 by PMA, it is possible that nPKCs, especially PKCδ, play a role in the induction of MCT4 by PMA in RD cells.

3.3. *Effects of specific PKC inhibitors on PMA-induced stimulation of MCT4 promoter activity in RD cells*

We next examined MCT4 promoter activity to determine whether the increase in MCT4 mRNA levels was a consequence of transcriptional activation of MCT4 in response to PMA. MCT4 promoter-luciferase construct was transiently transfected into RD cells and luciferase activity was measured at various times after the addition of PMA. At 24 h after PMA addition, there was clearly an increase in luciferase activity from the MCT4 promoter construct, which increased further after 48 h (Supplemental Fig. 2). These results suggest that chronic exposure to PMA stimulates MCT4 expression by increasing transcriptional activation of the MCT4 gene. To further confirm the involvement of PKC in PMA-induced MCT4 promoter activity, we performed experiments on inhibition of PKC. As in the case of protein levels, PMA-induced MCT4 promoter activity was inhibited by BIM and ROT (Table 1). Moreover,

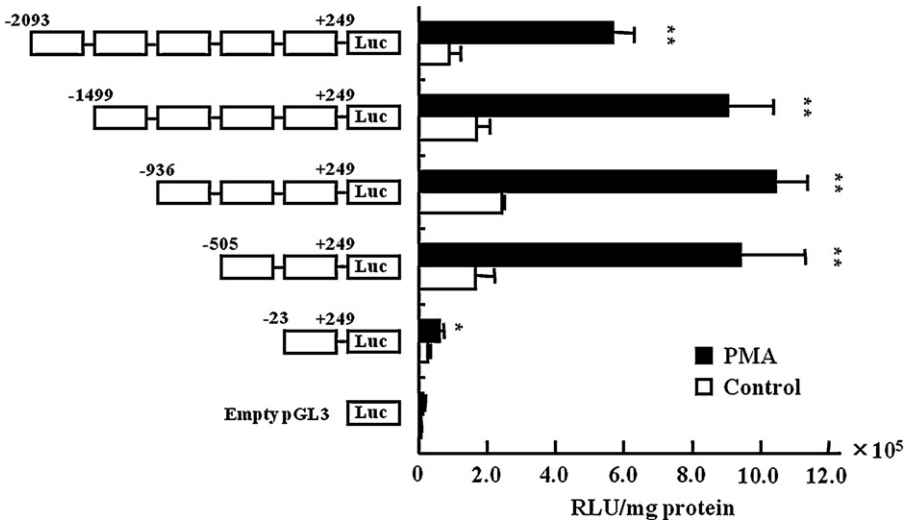

Fig. 3. Functional analysis of various deletion constructs in response to PMA. RD cells were transiently transfected with different 5′-deletion constructs of the MCT4 promoter as described above. After transfection, cells were treated with PMA (0.1 μM) for 48 h. The promoter activity was assessed and expressed as relative light units (RLU)/mg of protein. Each column represents the mean with S.D. of 3 determinations. *, **; significantly different from the control at $P < 0.05$, $P < 0.01$.

Table 1
Effects of PKC inhibitors on PMA-induced MCT4 promoter activity in RD cells.

Treatment	MCT4 promoter activity (% of control)
Control	100.0 ± 51.8
PMA (0.1 μM)	575.6 ± 40.9*
PMA (0.1 μM) + BIM (1 μM)	168.9 ± 44.0†
PMA (0.1 μM) + ROT (5 μM)	288.5 ± 57.6†

RD cells were tansfected with an MCT4 luciferase promoter construct (p−2093/+249).
After transfection, RD cells were exposed to PMA (0.1 μM) in the absence or presence of bisindolylmaleimide (BIM) and rottlerin (ROT) for 48 h and the promoter activity was assessed. Values represent means ± S.D. of 3 determinations.
* Significantly different from control at $P < 0.01$.
† Significantly different from 0.1 μM PMA alone at $P < 0.05$.

siRNA for PKCδ tended to decrease PKCδ protein level in RD cells compared with that in cells transfected with nontargeting siRNA and the PMA-induced MCT4 protein level in RD cells inhibited by PKCδ siRNA (data not shown). These results provide evidence for a role of PKCδ in mediating the effects of PMA on MCT4 expression. On the other hand, we examined the effect of PMA on PKCδ protein expression in RD cells. Following exposure to 0.1 μM PMA for 24 h, the levels of PKCδ protein tended to be decrease compared to that of non-treated cells by Western blot analysis (Supplemental Fig. 3). Johnson et al. reported that following prolonged exposure to 0.1 μM PMA for 48 h, the levels of PKCδ was reduced (Johnson et al., 1995). Our result was associated with this previous report. The reason for the increase of MCT4 expression after 0.1 μM PMA treatment for 24 h although the same PMA treatment decreased PKCδ expression might be so that PKCδ retains its activity while follows by down-regulation of the protein in return for PKCδ activation. Further investigations to examine the PKCδ translocation after PMA treatment are in progress. Chronic exposure of RD cells to PMA stimulates MCT4 mRNA and protein expression, which is associated with transcriptional activation of the MCT4 gene promoter. Additionally, the increase in MCT4 expression seen in this study is not due to initial PKC activation and could be a secondary effect following PKC activation by PMA.

Supplementary material related to this article found, in the online version, at doi:10.1016/j.ijpharm.2012.02.021.

3.4. Identification of regions in the MCT4 promoter that are critical for PMA-induced transcription in RD cells

In the next experiments, to determine which region of the MCT4 promoter is responsible for PMA-induced stimulation of MCT4 promoter activity, a series of MCT4 deletion constructs were transfected into RD cells, and luciferase activity was measured. The full-length promoter construct (p−2093/+249) exhibited approximately six-fold activation in promoter activity after PMA treatment compared with the control. Similarly, deletion constructs p−1499/+249, p−936/+249 and p−505/+249 showed an approximately four- to six-fold increase after PMA treatment. On the other hand, deleting nucleotides −505 to −24 significantly decreased both the basal and PMA-induced promoter activity (Fig. 3). These results suggested that the region between −505 and −24 was important for basal promoter activity and contained a PMA-responsive element. We performed a computational sequence analysis of this region using TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html). This region contained numerous GC-rich sites that supposedly bind with the transcription factor Sp1, and nuclear factor (NF)-κB binding site. Moreover, others have revealed the presence of two hypoxia-response elements (HREs) for MCT4 transcriptional regulation in this promoter region (Fig. 4A). Previous studies have shown the effect of hypoxia on MCT4 expression (Py et al., 2005; van der Meer et al., 2005) and the MCT4 promoter contains two potential HREs (Ullah et al., 2006). To define the contribution of these sites to PMA-induced MCT4 promoter activity, we performed several experiments involving the mutagenesis of these regions. We analyzed the effect of mithramycin A, a drug known to modify GC-rich regions of the DNA and to inhibit Sp1 binding (Ray et al., 1989; Blume et al., 1991). Inhibition of Sp1 binding by mithramycin A failed to abolish the stimulatory effects of PMA on MCT4 promoter activity (data not shown), indicating that Sp1 does not mediate the stimulatory effect of PMA on MCT4 transcription. Next, to determine whether NF-κB and HREs are responsible for stimulation of MCT4 by PMA, we introduced a mutation at these sites of the MCT4 (−505/+249) construct. When HRE site A was mutated, stimulation of MCT4 promoter activity by PMA was decreased by only 20%. When both site A and site B were mutated, PMA-induced MCT4 promoter activity was decreased by 60% (Fig. 4B). On the other hand, mutations in the potential NF-κB binding site exhibited no effect on the stimulation

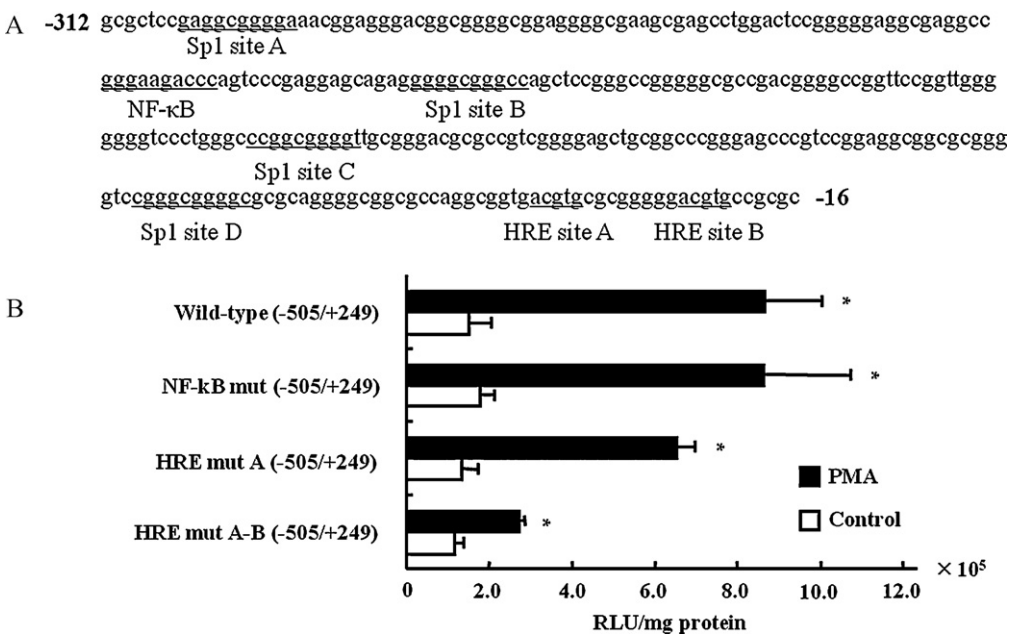

Fig. 4. Mutational analysis of putative NF-κB binding site and HREs within the MCT4 promoter. (A) The nucleotide sequence of the promoter region from −312 to −16 is shown with the putative NF-κB binding site and HREs (site A and site B). (B) RD cells were transiently transfected with MCT4 promoter constructs (p−505/+249 wild-type) or mutated constructs (p−505/+249 NF-κB mut, HRE mutA and HRE mutA–B). After transfection, cells were treated with PMA for 48 h. The promoter activity was assessed and expressed as relative light units (RLU)/mg of protein as described in Section 2. Each column represents the mean with S.D. of 3–6 determinations. *; significantly different from control at $P < 0.01$.

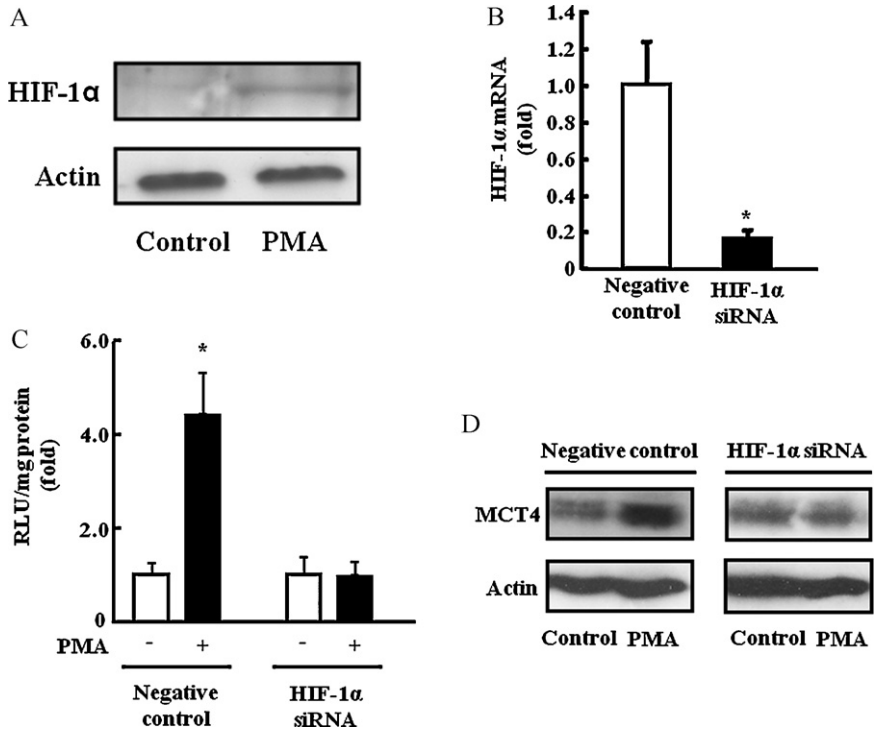

Fig. 5. Effects of PMA on HIF-1α protein level (A) and effects of HIF-1α siRNA on PMA-induced MCT4 promoter activity and protein levels (B–D) in RD cells. (A) RD cells were exposed to PMA (0.1 μM) for 48 h. Western blotting was carried out with antibodies to HIF-1α or β-actin as described above. Two separate experiments gave similar results. (B) Analysis of siRNA-mediated silencing of HIF-1α mRNA expression by quantitative real-time RT-PCR at 72 h after transfection. Each column represents the mean with S.D. of 3 determinations. *; significantly different from negative control siRNA at $P < 0.01$. (C) Effect of PMA on MCT4 promoter activity in negative control siRNA- and HIF-1α siRNA-treated RD cells. RD cells were transfected with negative control or HIF-1α siRNA (10 nM) for 72 h and treated with PMA (0.1 μM) for 48 h. Each column represents the mean with S.D. of 3–4 determinations. *; significantly different from the absence of PMA at $P < 0.01$. (D) Effect of PMA on MCT4 protein levels in negative control siRNA- and HIF-1α siRNA-treated RD cells. RD cells were transfected with negative control or HIF-1α siRNA (10 nM) for 72 h and treated with PMA (0.1 μM) for 24 h. Western blotting was carried out with antibodies to MCT4 or β-actin as described above. Two separate experiments gave similar results.

of MCT4 promoter activity by PMA (Fig. 4B). These results suggest that a transcriptional factor(s) that is up-regulated by hypoxia plays a major role in mediating the stimulatory effects of PMA on MCT4 promoter activity.

3.5. Contribution of HRE sites to PMA-induced MCT4 promoter activity

We further investigated the role of HIF-1α in mediating the effects of PMA on MCT4 expression and promoter activity. So we examined the effects of PMA on HIF-1α expression level in RD cells. A significant increase in HIF-1α protein level was seen when the cells were incubated with PMA (Fig. 5A). Moreover, the effect of HIF-1α knockdown on PMA-induced MCT4 promoter activity and protein level was investigated. SiRNA for HIF-1α significantly decreased HIF-1α mRNA level in RD cells compared with that in cells transfected with nontargeting siRNA (Fig. 5B). The PMA-induced MCT4 promoter activity and protein level in RD cells were completely inhibited by HIF-1α siRNA (Fig. 5C and D). These results suggest that PMA-induced MCT4 promoter activity and expression is regulated by an HIF-1α-dependent pathway.

The present study suggests the involvement of PKC signaling in a novel pathway associated with expression of MCT4. We have provided evidence that the effects of PMA on MCT4 expression are mediated by PKCδ. PKCδ is the most thoroughly studied member of the nPKC subfamily. Interestingly, previous studies have demonstrated that PKCδ is activated by hypoxia (Baek et al., 2001; Kim et al., 2004) and that it increases the protein stability and transcriptional activity of HIF-1α in human cancer cells (Lee et al., 2007). In another study, overexpression of PKCδ in primary skeletal muscle cells increased GLUT4 translocation and glucose uptake in the absence of insulin stimulation (Braiman et al., 1999). Moreover, others have proposed that muscle contraction-induced reduction in intracellular O_2 tension, by activating HIF-1α, might enhance transcription of the GLUT4 gene (Silva et al., 2005). Collectively, these findings suggest that PKCδ plays an important role in the regulation of muscle glycolytic metabolism and is involved in the regulation of HIF-1α-dependent gene in skeletal muscle.

The expression of MCT4 in muscle is regulated by a number of factors (Wang et al., 2003; Enoki et al., 2006, 2003). Muscle activity also has a strong influence on the expression of MCT4 (McDermott and Bonen, 1993; Pilegaard et al., 1999a; Bonen et al., 1998; Dubouchaud et al., 2000). MCT4 has been found to be up-regulated in skeletal muscle in a situation of limited O_2 availability such as during exercise (Green et al., 2002). Early studies indicated that PKC activation occurs during contraction of rat skeletal muscle (Richter et al., 1987). However, recent studies assessing isoform-specific PKC activation have failed to demonstrate an increase in cPKC or nPKC activity by contraction in human skeletal muscle (Perrini et al., 2004). Moreover, other studies have shown that acute exercise does not affect skeletal muscle PKCδ activity (Rose et al., 2004). Our results together with the above-mentioned observations support the view that PKCδ is an important signaling molecule involved in chronic adaptation to exercise, but not acute responses. However, further investigation is needed to determine whether activation of PKCδ accounts for the process of exercise-induced muscle adaptation.

4. Conclusions

In conclusion, our data demonstrated that expression of MCT4 in response to chronic PMA exposure is increased at the transcriptional level and that PKCδ plays a role in PMA-induced up-regulation of the MCT4 promoter in RD cells. We also showed the role of HIF-1α in PMA-induced up-regulation of the MCT4 promoter. Our findings suggest that PKCδ and HIF-1α can be involved in the up-regulation of MCT4 expression in RD cells and are therefore of significant importance for a better understanding of the molecular regulation of lactic acid metabolism in human skeletal muscle.

Acknowledgment

This work was supported in part by the Sasagawa Scientific Research Grant from The Japan Science Society.

References

Baek, S.H., Lee, U.Y., Park, E.M., Han, M.Y., Lee, Y.S., Park, Y.M., 2001. Role of protein kinase Cdelta in transmitting hypoxia signal to HSF and HIF-1. J. Cell Physiol. 188, 223–235.

Benton, C.R., Campbell, S.E., Tonouchi, M., Hatta, H., Bonen, A., 2004. Monocarboxylate transporters in subsarcolemmal and intermyofibrillar mitochondria. Biochem. Biophys. Res. Commun. 323, 249–253.

Benton, C.R., Yoshida, Y., Lally, J., Han, X.X., Hatta, H., Bonen, A., 2008. PGC-1alpha increases skeletal muscle lactate uptake by increasing the expression of MCT1 but not MCT2 or MCT4. Physiol. Genomics 35, 45–54.

Blume, S.W., Snyder, R.C., Ray, R., Thomas, S., Koller, C.A., Miller, D.M., 1991. Mithramycin inhibits SP1 binding and selectively inhibits transcriptional activity of the dihydrofolate reductase gene in vitro and in vivo. J. Clin. Invest. 88, 1613–1621.

Bonen, A., Heynen, M., Hatta, H., 2006. Distribution of monocarboxylate transporters MCT1-8 in rat tissue and human skeletal muscle. Appl. Physiol. Nutr. Metab. 31, 31–39.

Bonen, A., McCullagh, K.J., Putman, C.T., Hultman, E., Jones, N.L., Heigenhauser, G.J., 1998. Short-term training increases human muscle MCT1 and femoral venous lactate in relation to muscle lactate. Am. J. Physiol. 274, E102–E107.

Bonen, A., Miskovic, D., Tonouchi, M., Lemieux, K., Wilson, M.C., Marette, A., Halestrap, A.P., 2000a. Abundance and subcellular distribution of MCT1 and MCT4 in heart and fast-twitch skeletal muscles. Am. J. Physiol. Endocrinol. Metab. 278, E1067–E1077.

Bonen, A., Tonouchi, M., Miskovic, D., Heddle, C., Heikkila, J.J., Halestrap, A.P., 2000b. Isoform-specific regulation of the lactate transporters MCT1 and MCT4 by contractile activity. Am. J. Physiol. Endocrinol. Metab. 279, E1131–E1138.

Braiman, L., Alt, A., Kuroki, T., Ohba, M., Bak, A., Tennenbaum, T., Sampson, S.R., 1999. Protein kinase Cdelta mediates insulin-induced glucose transport in primary cultures of rat skeletal muscle. Mol. Endocrinol. 13, 2002–2012.

Broer, S., Schneider, H.P., Broer, A., Rahman, B., Hamprecht, B., Deitmer, J.W., 1998. Characterization of the monocarboxylate transporter 1 expressed in Xenopus laevis oocytes by changes in cytosolic pH. Biochem. J. 333, 167–174.

Cleland, P.J., Appleby, G.J., Rattigan, S., Clark, M.G., 1989. Exercise-induced translocation of protein kinase C and production of diacylglycerol and phosphatidic acid in rat skeletal muscle in vivo. Relationship to changes in glucose transport. J. Biol. Chem. 264, 17704–17711.

Cuff, M.A., Shirazi-Beechey, S.P., 2002. The human monocarboxylate transporter, MCT1: genomic organization and promoter analysis. Biochem. Biophys. Res. Commun. 292, 1048–1056.

Dempsey, E.C., Newton, A.C., Mochly-Rosen, D., Fields, A.P., Reyland, M.E., Insel, P.A., Messing, R.O., 2000. Protein kinase C isozymes and the regulation of diverse cell responses. Am. J. Physiol. Lung Cell Mol. Physiol. 279, L429–L438.

Denis-Pouxviel, C., Gauthier, T., Daviaud, D., Murat, J.C., 1990. Phosphofructokinase 2 and glycolysis in HT29 human colon adenocarcinoma cell line. Regulation by insulin and phorbol esters. Biochem. J. 268, 465–470.

Dimmer, K.S., Friedrich, B., Lang, F., Deitmer, J.W., Broer, S., 2000. The low affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. Biochem. J. 350, 219–227.

Dubouchaud, H., Butterfield, G.E., Wolfel, E.E., Bergman, B.C., Brooks, G.A., 2000. Endurance training, expression, and physiology of LDH, MCT1, and MCT4 in human skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 278, E571–E579.

Enoki, T., Yoshida, Y., Hatta, H., Bonen, A., 2003. Exercise training alleviates MCT1 and MCT4 reductions in heart and skeletal muscles of STZ-induced diabetic rats. J. Appl. Physiol. 94, 2433–2438.

Enoki, T., Yoshida, Y., Lally, J., Hatta, H., Bonen, A., 2006. Testosterone increases lactate transport, monocarboxylate transporter (MCT) 1 and MCT4 in rat skeletal muscle. J. Physiol. 577, 433–443.

Green, H., Halestrap, A., Mockett, C., O'Toole, D., Grant, S., Ouyang, J., 2002. Increases in muscle MCT are associated with reductions in muscle lactate after a single exercise session in humans. Am. J. Physiol. Endocrinol. Metab. 282, E154–E160.

Halestrap, A.P., Meredith, D., 2004. The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. Pflugers Arch. 447, 619–628.

Jeong, D., Kim, T.S., Lee, J.W., Kim, K.T., Kim, H.J., Kim, I.H., Kim, I.Y., 2001. Blocking of acidosis-mediated apoptosis by a reduction of lactate dehydrogenase activity through antisense mRNA expression. Biochem. Biophys. Res. Commun. 289, 1141–1149.

Johnson, J.A., Adak, S., Mochly-Rosen, D., 1995. Prolonged phorbol ester treatment down-regulates protein kinase C isozymes and increases contraction rate in neonatal cardiac myocytes. Life Sci. 57, 1027–1038.
Juel, C., Halestrap, A.P., 1999. Lactate transport in skeletal muscle—role and regulation of the monocarboxylate transporter. J. Physiol. 517, 633–642.
Kim, M.J., Moon, C.H., Kim, M.Y., Kim, K.H., Lee, S.H., Baik, E.J., Jung, Y.S., 2004. Role of PKC-delta during hypoxia in heart-derived H9c2 cells. Jpn. J. Physiol. 54, 405–414.
Knudsen, E.S., Pazzagli, C., Born, T.L., Bertolaet, B.L., Knudsen, K.E., Arden, K.C., Henry, R.R., Feramisco, J.R., 1998. Elevated cyclins and cyclin-dependent kinase activity in the rhabdomyosarcoma cell line RD. Cancer Res. 58, 2042–2049.
Kobayashi, M., Fujita, I., Itagaki, S., Hirano, T., Iseki, K., 2005. Transport mechanism for L-lactic acid in human myocytes using human prototypic embryonal rhabdomyosarcoma cell line (RD cells). Biol. Pharm. Bull. 28, 1197–1201.
Kobayashi, M., Otsuka, Y., Itagaki, S., Hirano, T., Iseki, K., 2006. Inhibitory effects of statins on human monocarboxylate transporter 4. Int. J. Pharm. 317, 19–25.
Lee, J.W., Park, J.A., Kim, S.H., Seo, J.H., Lim, K.J., Jeong, J.W., Jeong, C.H., Chun, K.H., Lee, S.K., Kwon, Y.G., Kim, K.W., 2007. Protein kinase C-delta regulates the stability of hypoxia-inducible factor-1 alpha under hypoxia. Cancer Sci. 98, 1476–1481.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Martiny-Baron, G., Kazanietz, M.G., Mischak, H., Blumberg, P.M., Kochs, G., Hug, H., Marmé, D., Schächtele, C., 1993. Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. J. Biol. Chem. 268, 9194–9197.
McCullagh, K.J., Juel, C., O'Brien, M., Bonen, A., 1996. Chronic muscle stimulation increases lactate transport in rat skeletal muscle. Mol. Cell. Biochem. 156, 51–57.
McCullagh, K.J., Poole, R.C., Halestrap, A.P., Tipton, K.F., O'Brien, M., Bonen, A., 1997. Chronic electrical stimulation increases MCT1 and lactate uptake in red and white skeletal muscle. Am. J. Physiol. 273, E239–E246.
McDermott, J.C., Bonen, A., 1993. Endurance training increases skeletal muscle lactate transport. Acta Physiol. Scand. 147, 323–327.
Newton, A.C., 2003. Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. Biochem. J. 370, 361–371.
Perrini, S., Henriksson, J., Zierath, J.R., Widegren, U., 2004. Exercise-induced protein kinase C isoform-specific activation in human skeletal muscle. Diabetes 53, 21–24.
Pilegaard, H., Domino, K., Noland, T., Juel, C., Hellsten, Y., Halestrap, A.P., Bangsbo, J., 1999a. Effect of high-intensity exercise training on lactate/H+ transport capacity in human skeletal muscle. Am. J. Physiol. 276, E255–E261.
Pilegaard, H., Juel, C., Wibrand, F., 1993. Lactate transport studied in sarcolemmal giant vesicles from rats: effect of training. Am. J. Physiol. 264, E156–E160.
Pilegaard, H., Terzis, G., Halestrap, A.P., Juel, C., 1999b. Distribution of the lactate/H+ transporter isoforms MCT1 and MCT4 in human skeletal muscle. Am. J. Physiol. 276, E843–E848.
Py, G., Eydoux, N., Lambert, K., Chapot, R., Koulmann, N., Sanchez, H., Bahi, L., Peinnequin, A., Mercier, J., Bigard, A.X., 2005. Role of hypoxia-induced anorexia and right ventricular hypertrophy on lactate transport and MCT expression in rat muscle. Metabolism 54, 634–644.
Ray, R., Snyder, R.C., Thomas, S., Koller, C.A., Miller, D.M., 1989. Mithramycin blocks protein binding and function of the SV40 early promoter. J. Clin. Invest. 83, 2003–2007.
Richter, E.A., Cleland, P.J., Rattiga, S., Clark, M.G., 1987. Contraction-associated translocation of protein kinase C in rat skeletal muscle. FEBS Lett. 217, 232–236.
Rose, A.J., Michell, B.J., Kemp, B.E., Hargreaves, M., 2004. Effect of exercise on protein kinase C activity and localization in human skeletal muscle. J. Physiol. 561, 861–870.
Saksena, S., Theegala, S., Bansal, N., Gill, R.K., Tyagi, S., Alrefai, W.A., Ramaswamy, K., Dudeja, P.K., 2009. Mechanisms underlying modulation of monocarboxylate transporter 1 (MCT1) by somatostatin in human intestinal epithelial cells. Am. J. Physiol. Gastrointest. Liver Physiol. 297, G878–G885.
Silva, J.L., Giannocco, G., Furuya, D.T., Lima, G.A., Moraes, P.A., Nachef, S., Bordin, S., Britto, L.R., Nunes, M.T., Machado, U.F., 2005. NF-kappaB, MEF2A, MEF2D and HIF1-a involvement on insulin- and contraction-induced regulation of GLUT4 gene expression in soleus muscle. Mol. Cell Endocrinol. 240, 82–93.
Smith, J.P., Drewes, L.R., 2006. Modulation of monocarboxylic acid transporter-1 kinetic function by the cAMP signaling pathway in rat brain endothelial cells. J. Biol. Chem. 281, 2053–2060.
Ullah, M.S., Davies, A.J., Halestrap, A.P., 2006. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. J. Biol. Chem. 281, 9030–9037.
van der Meer, D.L., van den Thillart, G.E., Witte, F., de Bakker, M.A., Besser, J., Richardson, M.K., Spaink, H.P., Leito, J.T., Bagowski, C.P., 2005. Gene expression profiling of the long-term adaptive response to hypoxia in the gills of adult zebrafish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 289, R1512–R1519.
Wang, Y., Tonouchi, M., Miskovic, D., Hatta, H., Bonen, A., 2003. T3 increases lactate transport and the expression of MCT4, but not MCT1, in rat skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 285, E622–E628.
Wilson, M.C., Jackson, V.N., Heddle, C., Price, N.T., Pilegaard, H., Juel, C., Bonen, A., Montgomery, I., Hutter, O.F., Halestrap, A.P., 1998. Lactic acid efflux from white skeletal muscle is catalyzed by the monocarboxylate transporter isoform MCT3. J. Biol. Chem. 273, 15920–15926.