Regulation of the Expression and Activity of Glucose and Lactic Acid Metabolism-Related Genes by Protein Kinase C in Skeletal Muscle Cells

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Received February 14, 2013; accepted June 4, 2013

Protein kinase C (PKC) modulators are very attractive therapeutic targets in cancer. Since most cancer cells display increased glycolysis, elucidations of the effects of PKC activation on glycolysis is necessary for the development of effective medicine. In the present study, to clarify the role of PKC in the regulation of glycolysis, we examined the effect of phorbol 12-myristate 13-acetate (PMA), a PKC activator, on the expression and activity of glucose and lactic acid metabolism-related genes in human rhabdomyosarcoma cells (RD cells). In parallel to increases in glucose uptake and mRNA levels of glucose transporters (GLUTs) induced by PMA treatment for 6 h, the hexokinase (HK) mRNA level and activity were also significantly increased in RD cells. On the other hand, a significant increase in lactate dehydrogenase (LDH) mRNA level and activity was seen when the cells were incubated with PMA for 24 h, but not for 6 or 12 h, and was associated with lactic acid production. These effects by PMA treatment were markedly suppressed by Bisindolylmaleimide (BIM), a PKC inhibitor. Furthermore, chetomin, a hypoxia-inducible factor 1 (HIF-1) inhibitor, completely abrogated the increment of LDH mRNA level and activity as well as monocarboxylate transporter (MCT) 4, a lactic acid efflux transporter. In conclusion, we found that HK and LDH activity induced by PKC activation was associated with the glucose uptake and lactic acid level and that LDH and MCT4 are modulated by a common factor, HIF-1.

Key words glycolysis; protein kinase C; skeletal muscle

Glucose is widely recognized as a central compound in metabolism, and glucose homeostasis as a whole is highly regulated in physiology.[1)] Glucose utilization is related to the ability of glucose to transfer across the plasma membrane, a step in which glucose transporters (GLUTs) play an essential role.[2)] The absorbed glucose is then phosphorylated by hexokinase (HK) in the cytoplasm to form glucose-6-phosphate for metabolic utilization, the first step of glucose metabolism. Two isoforms of HK (type I and type II) are expressed in skeletal muscle.[3)] On the other hand, lactate dehydrogenase (LDH) mediates lactic acid production from glucose metabolites[4)] and human LDH—the M form, predominantly found in muscle; and the H form, found mainly in cardiac muscle.[5)] The lactic acid produced by LDH was released by monocarboxylate transporter (MCT) 4. MCT4 is thought to play a primary role in the efflux of lactic acid from muscle fibers, and its expression has been shown to be associated with indices of glycolytic capacities,[6)] Halestrap and Price reported that MCT4 might be of particular importance in organization that depends on high levels of glycolysis to comply their energy needs.[7)]

Protein kinase C (PKC) plays important roles in intracellular signaling involved in many cellular responses.[8)] PKC activation occurs during contractions of rat skeletal muscle.[9)] Moreover, there is evidence that some PKC isoforms have a role in exercise-mediated glucose transport.[10)] And PKC affected the glucose uptake and GLUTs expression in skeletal muscle cells,[11)] but the effects of PKC activation on the expression and function of glucose and lactic acid metabolism-associated genes have been obscure.

Therefore, we aimed to determine the effects of PKC activation on glucose uptake and amount of lactic acid in human rhabdomyosarcoma cells (RD cells) as an *in vitro* skeletal muscle model. We also explored the hypothesis that the PKC activator affects the expression of GLUTs, HK I, II, LDH-M and MCT4 by evaluating mRNA expression and function.

MATERIALS AND METHODS

Chemicals Bisindolylmaleimide (BIM), a PKC inhibitor, was obtained from Funakoshi (Tokyo, Japan). Phorbol 12-myristate 13-acetate (PMA), a PKC activator, oxamate, an LDH inhibitor, chetomin, an hypoxia-inducible factor 1 (HIF-1) inhibitor, and all other chemicals were obtained from SIGMA (St. Louis, MO, U.S.A.). All other reagents were the highest grade available and used without further purification.

Cell Culture Experiments were carried out using cultured RD cells. RD cells of the spindle-cell type were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). The RD cells were maintained in plastic culture flasks as described previously,[12)] and the cells were kept in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin–streptomycin, respectively, at 37°C under 5% CO_2.

Uptake Study Glucose transport was studied using uptake of non-metabolizable glucose analog [^{3}H]-2-deoxy-D-glucose (2-DG). After removal of the growth medium, cells were washed with transport buffer (25 mM D-mannitol, 137 mM NaCl, 5.37 mM KCl, 0.3 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 1.26 mM $CaCl_2$, 0.8 mM $MgSO_4$

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and 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), pH 7.4) and preincubated at 37°C for 10 min with 0.5 mL of transport buffer. Uptake was initiated by applying transport buffer containing [^{3}H]-2-DG (0.2 μCi/mL). The uptake study was performed at 37°C for 20 min. Uptake was terminated by suctioning off the applied solution and immersing the plates in ice-cold transport buffer. To quantify the radioactivity of [^{3}H]-2-DG taken up by the cells, the cells were solubilized in 1% sodium dodecyl sulfate (SDS)/0.2 N NaOH. The remainder of the sample was mixed with 5 mL of scintillation cocktail to measure the radioactivity in a liquid scintillation counter.

Quantitative Real-Time Polymerase Chain Reaction (PCR) Quantitative real-time PCR was performed using an Mx3000TM Real-time PCR System with GoTaq® qPCR Master Mix as per the manufacturer's protocol and specific primers (sequences shown in Table 1) through 40 cycles of 95°C for 30 s, 50°C (HKI, HKII, and LDH-M), 55°C (GLUT1 and GLUT3), and 60°C (MCT4) for 30 s, and 72°C for 1 min. The PCR products were normalized to amplified 18S, which was the internal reference.

Enzymatic Activity Assays HK and LDH activity was measured spectrophotometrically. HK activity assays were performed at 30°C in a reaction medium containing 50 mM Tris–HCl (pH 7.6), 8.3 mM D-glucose, 10.3 mM ATP, 20.7 mM $MgCl_2$, 0.1 mM β-nicotinamide adenine dinucleotide phosphate (NADP), 1.7 U/mL glucose-6-phosphate dehydrogenase and cell extracts. After 3-min intervals, activities were measured at 340 nm. The enzyme activity was expressed as m-units of HK activity. LDH activity assays were performed at 30°C in a reaction medium containing 50 mM Tris–HCl (pH 7.6), 40 μM NADH, 2.5 mM sodium pyruvate and cell extracts. After 1-min intervals, activities were measured at 340 nm. The enzyme activity was expressed as m-units of LDH activity.

Determination of Lactic Acid Content Lactic acid content in the supernatant was measured spectrophotometrically using a lactic acid reagent (Trinity Biotech U.S.A., St. Louis, MO, U.S.A.) as per the manufacturer's protocol.

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 Turkey test. Statistical significance was defined as $p<0.05$.

RESULTS AND DISCUSSION

Effects of PKC Activator and Inhibitor on the Expression and Activity of Glucose Metabolism-Related Genes in RD Cells Firstly, we investigated the effect of PMA, a PKC activator, on the uptake of 2-DG in RD cells. As shown in Fig. 1A, PMA treatment for 20 min to 24 h increased 2-DG uptake, particularly at 6 h. Moreover, 2-DG was transported in a concentration-dependent manner. A comparison of the apparent dissociation constants (K_m) and maximum rates of uptake (V_{max}) in control and PMA-treated cells showed that V_{max} in PMA-treated cells was larger than that in control cells, whereas K_m was not significantly altered (Fig. 1B). In parallel to an increase in 2-DG uptake, PMA increased GLUT1, 3 mRNA levels (Fig. 1C), but not that of GLUT4 (data not shown). BIM, a specific PKC inhibitor, blocked the PMA-induced 2-DG uptake and GLUT1, 3 mRNA levels (Fig. 1D, Supplemental Table 1). Previous studies have shown that BIM at a concentration of 1 μM is sufficient to inhibit most of the PKC isoforms.[13] Therefore, these results indicated that the effect of PMA was mediated by stimulation of PKC. To clarify the role of PKC in the expression and function of glucose metabolism-related genes, the effects of PMA and BIM on HK mRNA levels and activity were investigated. PMA increased HK I mRNA levels at 6, 12 and 24 h. On the other hand, HK II mRNA was greatly increased at 6 h after PMA treatment and then decreased to the control level in a time-dependent manner. These effects were markedly suppressed by BIM (Figs. 2A, B). The expression of HK II isozyme in skeletal muscle can vary significantly depending on physical activity and hormonal status. The content of HK II mRNA in rat skeletal muscle also increases after infusion of insulin.[14,15] In contrast to these patterns with HK II, the activity and expression of the HK I isozyme are relatively insensitive to hormonal influence. We speculate that the difference in the response to PMA is caused by the difference in the roles of such HK isozymes. HK activity was increased notably 6 h after PMA addition (Table 2) and the increases was associated with HK mRNA levels.

Regulation of the Expression and Activity of Lactic Acid Metabolism-Related Genes by PKC and HIF-1 in RD Cells Next, since LDH mediates lactic acid production from glucose that has been transferred to skeletal muscle cells, it is possible

Table 1. Primer Information

Gene	Gene bank accession No.		Sequences
GLUT1	NM_006516	Forward	5′-TCA CTG TGC TCC TGG TTC TG-3′
		Reverse	5′-CCT GTG CTC CTG AGA GAT CC-3′
GLUT3	NM_006931	Forward	5′-ACC GGC TTC CTC ATT ACC TT-3
		Reverse	5′-AGG CTC GAT GCT GTT CAT CT-3′
HKI	NM_000188	Forward	5′-CTG AAT AGC ACC TGC GAT GA-3′
		Reverse	5′-ACA TTC AGA CGG TCC AGT CC-3′
HKII	NM_000189	Forward	5′-TTG AGA GCA CCT GTG ACG AC-3′
		Reverse	5′-CCA CAC CCA CTG TCA CTT TG-3′
LDH-M	NM_005566	Forward	5′-TGT GCC TGT ATG GAG TGG AA-3′
		Reverse	5′-AGC ACT CTC AAC CAC CTG CT-3′
MCT4	NM_004207	Forward	5′-ATT GGC CTG GTG CTG CTG ATG-3′
		Reverse	5′-CGA GTC TGC AGG AGG CTT GTG-3′
18S	NR_003286	Forward	5′-CGG CTA CCA CAT CCA AGG AA-3′
		Reverse	5′-GCT GGA ATT ACC GCG GCT-3′

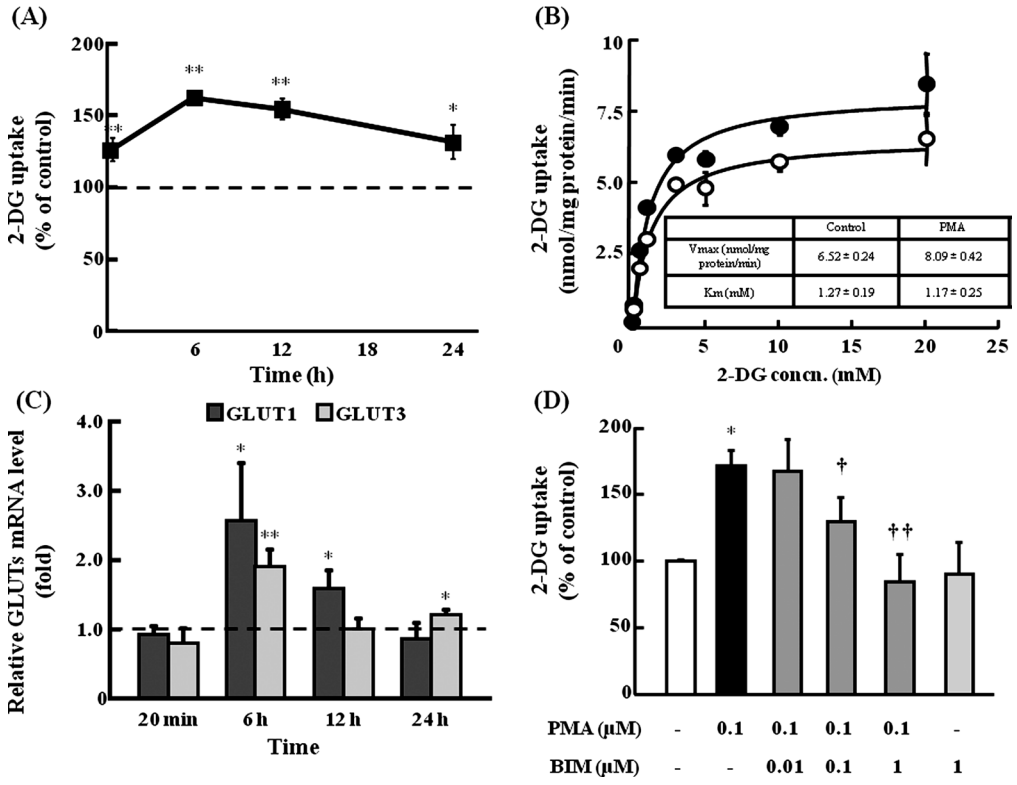

Fig. 1. Effects of PMA on 2-DG Uptake (A), Concentration Dependent 2-DG Uptake (B), mRNA Levels of GLUTs (C) and Inhibitory Effects of BIM on PMA-Induced 2-DG Uptake (D) in RD Cells

(A) RD cells were exposed to PMA (0.1 μM) for 20 min, 6, 12 and 24 h. Data (n=3) are given as means±S.D. and shown are typical results. *p<0.05 or **p<0.01 compared with vehicle control. (B) RD cells were exposed to 0.1 μM PMA (●) or DMSO (control; ○) for 6 h. The points (n=3–6) are given as means±S.D. of more than two independent experiments. (C) RD cells were exposed to PMA (0.1 μM) for 20 min, 6, 12 and 24 h. Data (n=3) are given as means with S.D. and shown are typical results. *p<0.05 or **p<0.01 compared with vehicle control. (D) RD cells were exposed to PMA (0.1 μM) in the absence or presence of varying concentrations of BIM for 6 h. Bar graphs (n=3–6) are given as means with S.D. of more than two independent experiments. *p<0.01 compared with vehicle control. $^{\dagger}p$<0.05 or $^{\dagger\dagger}p$<0.01 compared with PMA.

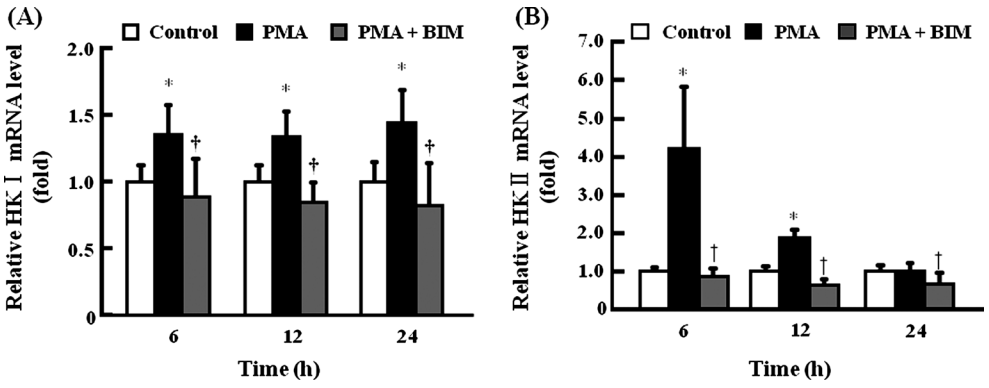

Fig. 2. Effects of PKC Modulators on mRNA Levels of HK I (A) and HK II (B) in RD Cells

RD cells were exposed to PMA (0.1 μM) in the absence or presence of BIM (1 μM) for 6, 12 and 24 h. Bar graphs (n=6–18) are given as means with S.D. of more than two independent experiments. *p<0.01 compared with vehicle control. $^{\dagger}p$<0.01 compared with PMA.

Table 2. Effects of PKC Modulators on HK and LDH Activities in RD Cells

	HK activity (mU/mg protein)				LDH activity (mU/mg protein)			
	Control	PMA	PMA+BIM	BIM	Control	PMA	PMA+BIM	BIM
6 h	49.1±3.6	59.2±4.2**	52.2±4.2†	52.6±5.0	212±14.0	212±15.8		—
12 h	50.7±4.4	57.6±6.7*	54.2±1.0	55.4±1.0	207±20.9	209±25.3	—	—
24 h	53.8±6.7	57.9±2.6	—	—	211±15.7	233±11.5**	205±10.3††	203±3.2

RD cells were exposed to PMA (0.1 μM) in the absence or presence of BIM (1 μM) for various periods of time (6, 12 and 24 h). HK and LDH activities were assessed and expressed as m-units (mU)/mg of protein as described in Materials and Methods. Data (n=3–15) are given as means±S.D. of more than two independent experiments. *p<0.05 or **p<0.01 compared with vehicle control. $^{\dagger}p$<0.05 or $^{\dagger\dagger}p$<0.01 compared with PMA.

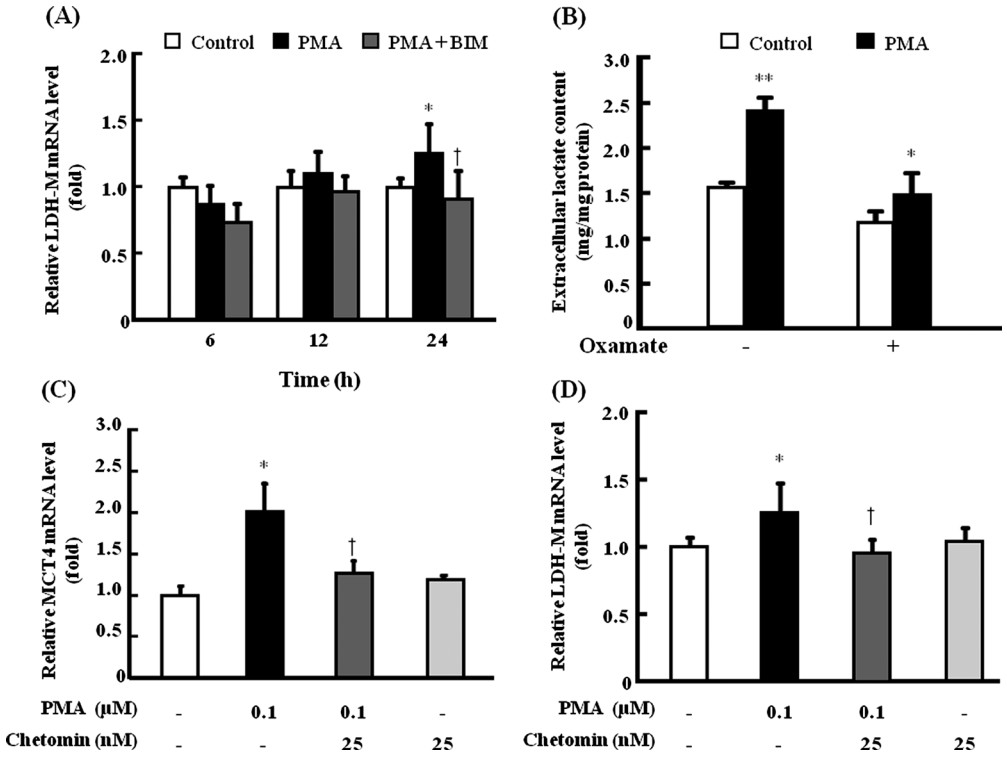

Fig. 3. Effect of PKC Modulators on LDH-M mRNA Level (A), Effect of LDH Inhibitor on PMA-Induced Lactic Acid Secretion (B) and Effect of HIF-1 Inhibitor on PMA-Induced MCT4 (C) and LDH-M (D) mRNA Levels in RD Cells

(A) RD cells were exposed to PMA (0.1 μM) in the absence or presence of BIM (1 μM) for 6, 12 and 24 h. Bar graphs (n=6–18) are given as means with S.D. of more than two independent experiments. *p<0.01 compared with vehicle control. $^{\dagger}p$<0.01 compared with PMA. (B) RD cells were exposed to PMA (0.1 μM) in the absence or presence of oxamate (20 mM) for 24 h. Bar graphs (n=3–6) are given as means with S.D. of more than two independent experiments. *p<0.05 or **p<0.01 compared with vehicle control. (C, D) RD cells were exposed to PMA (0.1 μM) in the absence or presence of chetomin (25 nM) for 24 h. Bar graphs (n=6–18) are given as means with S.D. of more than two independent experiments. *p<0.01 compared with vehicle control. $^{\dagger}p$<0.01 compared with PMA.

Table 3. Inhibitory Effect of Chetomin on PMA-Induced LDH Activity in RD Cells

LDH activity (mU/mg protein)			
Control	PMA	PMA+Chetomin	Chetomin
211±15.7	233±11.5*	206±16.9†	206±14.3

RD cells were exposed to PMA (0.1 μM) in the absence or presence of chetomin (25 nM) for 24 h. Data (n=12–15) are given as means±S.D. of more than two independent experiments. *p<0.01 compared with vehicle control. $^{\dagger}p$<0.01 compared with PMA.

that LDH expression and function are enhanced in response to PMA treatment. The effects of PKC modulators on LDH mRNA and activity were therefore examined. A significant increase in LDH mRNA was seen when cells were incubated with PMA for 24 h but not when cells were incubated with PMA for 6 or 12 h (Fig. 3A). Parallel to the increase in LDH mRNA levels in response to PMA, 24-h incubation with 0.1 μM PMA significantly increased LDH activity (Table 2). Figure 3B shows that the PMA-induced lactic acid release from RD cells was significantly inhibited by oxamate, an LDH inhibitor. These results suggest that PMA increased lactic acid production mediated by LDH. A previous study showed that exposure of RD cells to PMA resulted in a significant increase in MCT4 mRNA level from 12 to 24 h and HIF-1α protein level was increased in a time-dependent manner.[16] It has also been shown that PKC is activated by hypoxia[17] and that PKC increases the protein stability and transcriptional activity of HIF-1α in human cancer cells.[18] We hypothesized that the expression of LDH and that of MCT4 are regulated by a common factor, HIF-1. As shown in Figs. 3C, D and Table 3, in the presence of chetomin, an inhibitor of HIF-1, the stimulatory effects of PMA were abolished. These results suggest that PKC and HIF-1 can be involved in the induction of LDH and MCT4 by PMA in RD cells. Increased PKC expression and activity have been demonstrated in many cancers.[19,20] PKCs may play important roles in tumor formation and progression, invasiveness of cancer cells, and chemoresistance.[21,22] Shime *et al.* previously decribed that lactic acid secreted by tumor cells enhances the production of interleukin (IL)-23 by monocytes/macrophages and IL-23 was overexpressed in and around tumor tissues, where it induced local inflammation and promoted tumor development.[23] Recently, it was reported that lactic acid activated HIF-1 and triggers tumor angiogenesis and tumor growth.[24] Our results showed that PKC activation modulated glycolysis and lactic acid levels in RD cells. Collectively, we suggest that PKC inhibitor may lead to reduction in the chance of the rhabdomyosarcoma by inhibition of glycolysis and production of lactic acid.

In conclusion, our results showed that the expression and activity of HK and LDH induced by PMA exposure is associated with glucose and lactic acid levels. We also showed the role of HIF-1 in PMA-induced up-regulation of LDH. Our

findings suggest that PKC is involved in up-regulation of the expression and function of glucose and lactic acid metabolism-related genes in RD cells.

Acknowledgment This study was funded in part by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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