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Inflammation increases pyruvate dehydrogenase kinase 4 (PDK4) expression via the Jun N-Terminal Kinase (JNK) pathway in C2C12 cells

Hana Park [a], Nam Ho Jeoung [b, *]

[a] Department of Biomedical Sciences, Kyungpook National University School of Medicine, Daegu, Republic of Korea
[b] Department of Pharmaceutical Science and Technology, Catholic University of Daegu, Gyeongsan, Republic of Korea

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ABSTRACT

Chronic inflammation augments the deleterious effects of several diseases, particularly diabetes, cancer, and sepsis. It is also involved in the process of metabolic shift from glucose oxidation to lactate production. Although several studies suggest that the change in activity of the pyruvate dehydrogenase complex (PDC) is a major factor causing this metabolic change, the exact mechanism of the inflammatory state remains unclear. In this study, we investigated the effect of lipopolysaccharide (LPS) on the expression of pyruvate dehydrogenase kinase 4 (PDK4), which is strongly associated with inactivation of the PDC in C2C12 myoblasts.

In C2C12 myoblasts, LPS exposure led to increased PDK4 mRNA and protein expression levels as well as lactate production in culture medium. However, the expression levels of other PDK isoenzymes (PDK1 – 3) remained unchanged. Additionally, we observed that LPS treatment induced phosphorylation of Jun N-Terminal Kinases (JNK). To confirm the role of JNK, we inhibited the JNK pathway and observed that PDK4 expression and lactate production were decreased, but p38 and ERK were not significantly changed.

Taken together, our results suggest that LPS induces PDK4 expression and alters glucose metabolism via the JNK pathway.

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1. Introduction

Chronic inflammation caused by several diseases such as diabetes, cardiovascular disease, cancers, and sepsis augments the deleterious effect of the disease conditions. It induces insulin resistance and diabetic complications [1,2], atherosclerosis [3,4], cachexia and anorexia [5], as well as M2 polarization of macrophages [6]. Onset of this inflammation-induced change in phenotype is commonly associated with metabolic changes, specifically glucose metabolism.

Sepsis is a serious inflammatory response to infection by various microbes, and causes a metabolic syndrome [7]. Endotoxin or lipopolysaccharide (LPS) constitutes part of the outer membrane of gram-negative bacteria. Several cytokines such as TNF-α, IL-6, and IL-1β are secreted in response to endotoxin or LPS exposure, resulting in the onset of sepsis and deterioration [8–10]. Sepsis is found to be more exacerbating in children, the elderly, immunocompromised individuals, as well as those with AIDS, cancer, or diabetic conditions [8,11]. Fever, low blood pressure, and high blood lactate are common symptoms of sepsis conditions [12–14]. An increase in blood lactate levels suggests a close relationship between sepsis and glucose metabolic change.

The pyruvate dehydrogenase complex (PDC) promotes the decarboxylation of pyruvate into acetyl-CoA, which is regarded as a gate keeper enzyme [15] that connects glycolysis and the TCA cycle. PDC activity is regulated by pyruvate dehydrogenase kinase (PDK), which phosphorylates and inhibits PDC activity. Therefore, PDK is a crucial regulator of the PDC [16–21]. In the normal state, glucose is converted to pyruvate, and this is further converted to acetyl-coA by PDC for generating energy via the TCA cycle. However, in diseases states such as sepsis, pyruvate is converted to lactate, leading to an increase in blood lactate [15,22]. The lowered PDC activity presumably causes this phenotypic change. In cancer cells, PDC inactivation is caused by the increase in PDKs due to hypoxia inducible factor 1α (Hif-1α) stabilization [23]. This suggests that inflammation could also induce the expression of PDKs and inactivate PDC activity. However, this has not been fully elucidated.

* Corresponding author. Department of Pharmaceutical Science and Technology, Catholic University of Daegu, 13-13 Hayang-ro Hayang-eup, St. Thomas Build. Rm 419, Gyeongsan 38430, Republic of Korea.
E-mail address: syjeoung@cu.ac.kr (N.H. Jeoung).

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Four PDK isoforms (PDK1, 2, 3, and 4) have been identified in human and mammalian tissues [24,25]. Among them, PDK4 plays a pivotal role in the regulation of PDC activity in the skeletal muscle cell. It has been reported that peroxisome proliferator-activated receptor gamma (PPARγ) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) induce PDK4 expression, and glucocorticoid, thyroid, and growth hormones can upregulate PDK4 expression in skeletal muscles [16,26]. On the other hand, insulin represses PDK4 expression by phosphorylation of FOXO [27,28]. However, it is not clear yet how inflammation inhibits oxidative glucose metabolism, and which PDK isoenzyme is involved in PDC inactivation by inflammation.

In this study, we investigated the PDK isoenzymes to elucidate which of them is responsible for inhibiting PDC activity in mouse skeletal muscle in response to LPS induced inflammation. We found that LPS significantly induced PDK4 expression via activation of the Jun N-Terminal Kinase (JNK) pathway. These results therefore suggest that PDK4 is a potential therapeutic target for development of an anti-sepsis drug.

2. Materials and methods

2.1. Cell culture

Mouse C2C12 myoblast cells were purchased from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone, SH30919.03) at 37 °C in a humidified 5% CO_2 incubator. Cells were grown to confluence and fresh serum-containing media was added prior to addition of LPS and mitogen-activated protein kinase (MAPK) inhibitors or dichloroacetate (DCA). Cells were treated with LPS (Sigma–Aldrich, L2880), JNK inhibitor (Abcam, ab120065), ERK inhibitor (Abcam, ab120234), p38 inhibitor (Abcam, ab120638), or DCA (Sigma–Aldrich, 347795) and harvested and lysed for real-time PCR or western blotting.

2.2. Western blotting

Myoblasts were washed with PBS and lysed for 30 min on ice by using lysis buffer (50 mM Tris–HCl (pH8.0) containing 150 mM NaCl, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na2EDTA, 1 mM EGTA, 1 mM Na_3VO_4 and 1 mg/mL leupeptin). The cell extracts were subjected to a Bradford assay (Bio-rad, #500-0006) to determine protein concentration. Proteins were then separated on 15% SDS-PAGE and transferred to PVDF membranes (millipore, IPFL00010). Western blot analysis was performed according to the standard method with specific primary antibodies (1:1000 diluted in TBST buffer containing 0.5% Tween-20). Proteins were detected with the following primary antibodies: phospho-PDHE1α (Ser^{300}) (Calbiochem, AP1064), phospho-JNK (Cell signaling, #9251), JNK (Cell signaling, #9252), phospho-ERK (Cell signaling, #9101), ERK (Cell signaling, #9102), phospho-p38 (Cell signaling, #9215), p38 (Cell signaling, #9212), GAPDH (Santa cruz, sc-25778), and PDK4 antiserum (obtained from Dr. Robert A. Harris, Indiana University, Indianapolis, USA). The immunoblotted membranes were scanned according to the manufacturer's instructions and visualized (DNR, MicroChemi systems).

2.3. Quantitative real time (RT)-PCR

Total RNA was extracted from C2C12 myoblasts cells using RNAiso Plus (Takara, 9109) according to the manufacturer's instruction. RNA concentration was measured using a Nanodrop 2000 (maestrogen, MN-913). Total RNA was reverse-transcribed to single-strand cDNA using a cDNA synthesis kit (Thermo, K1622). RT-PCR was performed using the SYBR (Takara, RR420A) method. The following primer sequences were used in this study; PDK1 forward 5′-ACCAGCACTCCTTATTGTTCGGTGG-3′, PDK1 reverse 5′-TCACAAAGCCGCCTAGCGTTCTC-3′; PDK2 forward 5′-TTCTACCT-CAGCCGCATCTCCATC-3′, PDK2 reverse 5′-CTTTCACCACATCAGA-CACGCTGC-3′; PDK3 forward 5′-TGCGACTGGCTAACACCATGAGAG-3′, PDK3 reverse 5′-AAACTCGTGGGTCTTCTGGGCTC-3′; PDK4 forward 5′-TGTGGTAGCAGTAGTCCAAGATGCC-3′, PDK4 reverse 5′-GCATGTGGTGAAGGTGTGAAGGAAC-3′; β-actin forward 5′-CGCCACCAGTTCGCCATGGA-3′, β-actin reverse 5′-TACAGCCCGGG-GAGCATCGT-3′. The housekeeping gene β-actin was used as an internal control.

2.4. Measurement of lactate concentration

C2C12 myoblasts were treated with LPS, MAPK inhibitor, or DCA in RPMI-1640 serum free media. The culture media was collected 24 h after treatment. Collected samples were deproteinized using the 6% perchloric acid method. The supernatants were then collected and neutralized using 30% KOH. The lactate concentration was measured using a spectrophotometer [29] (SHIMADZU, UV-1800).

2.5. Statistical analysis

Results are expressed as mean ± SEM. Statistical significance was evaluated by Student's *t-test*. A p value < 0.05 was considered statistically significant.

3. Results

3.1. LPS exposure to C2C12 myoblasts alters the expression of pyruvate dehydrogenase kinases (PDKs)

We examined the effect of LPS induced inflammation on the expression of PDK isoenzymes in C2C12 myoblasts. LPS treatment significantly increased the mRNA expression of PDK4 (Fig. 1A). However, PDK2 mRNA expression was slightly but significantly reduced by LPS treatment, indicating that this isoenzyme is not involved in inflammation induced glucose metabolic alteration. However, the mRNA expression levels of PDK1 and PDK3 remained unchanged with LPS treatment. Since the expression of PDK4 was most significantly affected by LPS stimulation, we examined whether this effect was dose or time dependent. As expected, LPS treatment significantly induced PDK4 expression in a dose- and time-dependent manner (Fig. 1B and C, respectively). Similar results were also observed at the protein level (Fig. 1D).

3.2. LPS treatment increases lactate production in C2C12 myoblast

PDK4 is a main regulator of PDC activity in skeletal muscle, and it alters glucose metabolism [7]. Therefore, we examined the phosphorylation of PDHE1α, a marker of PDC inhibition by PDKs, and lactate production, a marker of glucose metabolic alteration from the oxidative pathway to lactate production [15]. LPS significantly increased the phosphorylation of PDHE1α(Ser^{300}) (Fig. 2A), which suggests that PDC is inactivated by LPS treatment. Lactate production was significantly increased by LPS treatment (Fig. 2B). These results indicate that inflammation can shift glucose metabolism from oxidative phosphorylation (OxPhos) to lactate production in C2C12 myoblasts cells.

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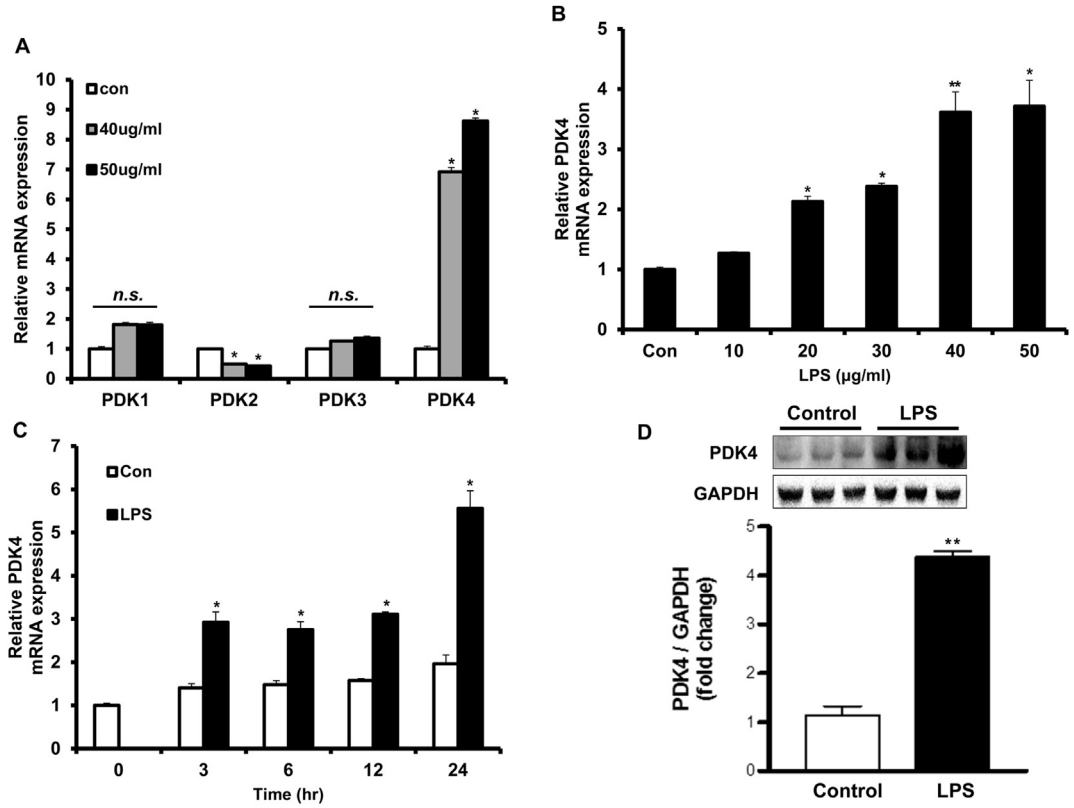

Fig. 1. LPS increases the expression of PDKs in C2C12 myoblasts. (A) Effect of LPS on mRNA expression of PDK isoforms. (B and C) Concentration- and time-dependent effects of LPS on PDK4 expression. PDK4 mRNA was measured by real-time PCR after treatment with the indicated concentration of LPS (B) for the indicated times (C) β-actin was used as a loading control. (D) Western blot analysis of PDK4 gene expression after LPS treatment (40 μM, 24hr). Values are means ± SEM. n = 3 per group. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control.

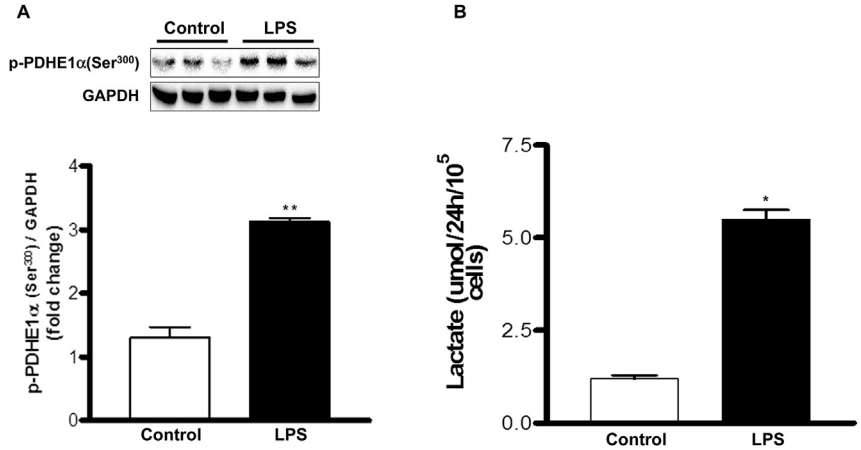

Fig. 2. LPS increases p-PDHE1α(Ser^{300}) and lactate production in C2C12 myoblasts after 40 μM LPS treatment for 24 h. (A) Phosphorylation of PDHE1α(Ser^{300}) was measured using a phospho-specific PDHE1α antibody. (B) Lactate production in the culture supernatant was measured. Values are means ± SEM. n = 3 per group. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control.

3.3. LPS increases PDK4 expression via the MAPK signaling pathway in C2C12 myoblasts

During sepsis, LPS-induced inflammation is initiated by LPS binding to Toll-like receptor 4 (TLR4), leading to activation of MAPKs such as JNK, ERK, and p38 [30]. In order to examine the effect of LPS on these pathways in C2C12 cells, we performed western blot analysis using specific antibodies for MAPKs. LPS treatment (40 μM) significantly increased phosphorylation of JNK, p38, and ERK (Fig. 3A), confirming the involvement of the MAPK pathway in C2C12 myoblasts after LPS treatment. This suggests that activation of MAPK may involve PDK4 expression.

Next, we investigated which MAPK was necessary for controlling PDK4 expression by LPS. In order to do this, we utilized specific MAPK inhibitors such as SP-600125 (JNK), PD-98059 (ERK), and SB-202190 (p38). We observed that inhibition of JNK dramatically suppressed LPS induced PDK4 expression, whereas ERK inhibition could not repress its expression. In addition, the p38 inhibitor

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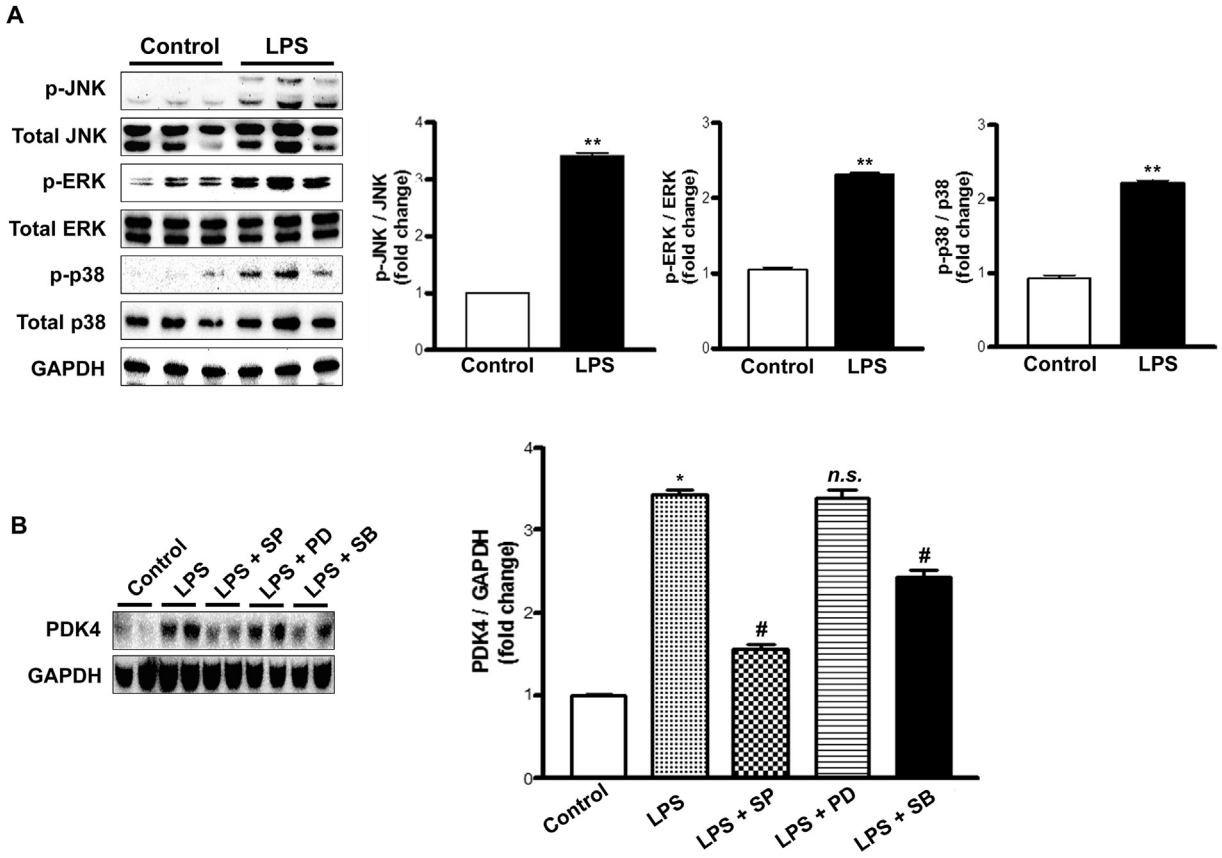

Fig. 3. Effect of LPS on activation of MAPK pathways in C2C12 myoblasts. (A) Phosphorylation of MAPKs were measured after treatment of 40 μM LPS for 30 min. (B) Effect of inhibition of MAPKs on the PDK4 expression. Cells were pre-treated with 20 μM SP-600125 (JNK inhibitor), PD-98059 (ERK inhibitor), and SB-202190 (p38 inhibitor) for 1 h. The cells were then stimulated for 30 min with 40 μM LPS. The protein expression of PDK4 was measured by Western blot analysis. Values are means ± SEM. n = 3 per group. **P < 0.01, *P < 0.05 vs. control, #P < 0.05 vs. LPS.

partially repressed LPS-induced PDK4 expression (Fig. 3B). This indicates that LPS activates JNK leading to increased PDK4 expression in C2C12, which could shift glucose metabolism from OxPhos to lactate formation.

3.4. JNK inhibition abrogates LPS-induced lactate production in C2C12 myoblasts

In order to verify that LPS-induced JNK activation is responsible for the metabolic switch of glucose, we examined the change in lactate production with JNK inhibition. Pre-treatment with a JNK inhibitor for 1 h significantly reduced LPS-induced lactate production for 24 h (Fig. 4A). Treatment with DCA, a well-known PDK4 inhibitor [31–33], also inhibited LPS-induced lactate production in C2C12 cells. This strongly suggests that the JNK pathway is engaged in alteration of glucose metabolism through increased PDK4 expression in C2C12 myoblasts.

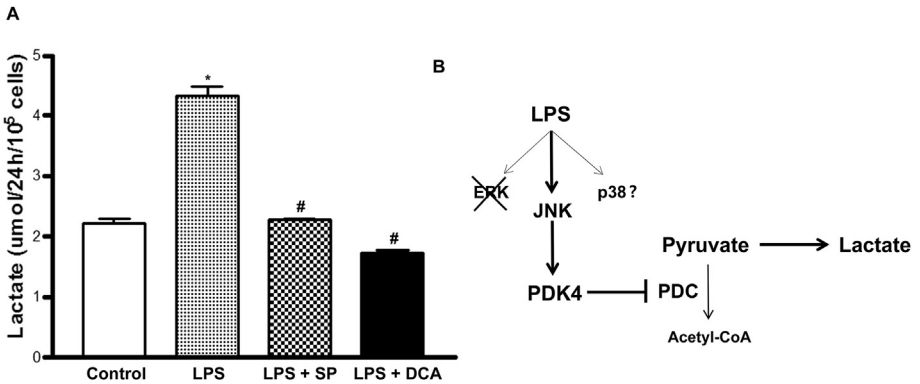

Fig. 4. Effect of JNK inhibition on lactate production in C2C12 myoblasts. (A) The concentration of lactate in the culture medium was measured. The cells were pre-treated with 20 μM SP-600125 (JNK inhibitor) or 25 mM DCA (PDK inhibitor), then treated with 40 μM LPS for another 24 h. Values are means ± SEM. n = 3 per group. *P < 0.05 vs. control, #P < 0.05 vs. LPS. (B) Schematic diagram of LPS-induced lactate production in C2C12 myoblasts.

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4. Discussion

Sepsis is an acute life threatening disease induced by bacterial infection. It has been reported that sepsis is responsible for increased levels of blood lactate, which induces lactoacidosis [34]. However, the mechanism responsible for the glucose metabolic switch from OxPhos to non-oxidative pathway, lactate fermentation, is not fully understood. In this study, we provide evidence that LPS induced the expression of PDK4 via activation of the JNK signaling pathway in C2C12 myoblasts. This inactivates PDC activity by phosphorylation of PDHE1α, leading to inhibition of OxPhos of glucose, which converts pyruvate, the end product of glycolysis, to lactate (Fig. 4B).

Exposure of skeletal muscle cells to endotoxin or LPS activates inflammatory signaling pathways, specifically TRAF6 dependent activation of NF-κB and MAPKs [30,35]. These transcription factors increase the expression of various cytokines including TNF-α, IL-1β, and IL-6 [36,37]. These cytokines induce the inflammatory state, leading to increased risk factors of insulin resistance, diabetes, hypertension, and cardiac vascular disease [38,39]. Inflammation also increases blood lactate levels, which could be due to alterations in cellular glucose metabolism. LPS increased the expression of PDK4 in C2C12 myoblasts, resulting in phosphorylation of PDHE1α, which possibly inactivates PDC activity. The phosphorylation state of PDHE1α strongly correlates with inactivation of PDC activity [40,41]. In addition, lactate production is increased in cancer cells due to the increase in PDKs, especially PDK1, by Hif1α [23]. These studies suggest that inactivation of PDC activity leads to lactate accumulation in cells. Our observation that LPS increased PDHE1α phosphorylation correlates with these previous reports. Activation of PDC activity by DCA significantly reduced the lactate concentration in the culture medium, indicating that inhibition of PDK is responsible for lactate production after activation of the inflammatory response. Conclusively, our result suggests that PDK4 is a suitable target for developing drugs against inflammation and sepsis.

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