Glucose Transporter Type 4 Redistribution on the Membrane Induced by Insulin through Akt in Hydrocortisone Treatment in Rat Skeletal Muscles

Chien-Min Chen1,2,3, Lian Chiu4, Hung-Chi Chen5, Chun-Yuan Cheng3, Woei-Cherng Shyu6,7, Chii-Wen Chou8, Cheng-You Lu6,7, and Chung-Tien Lin1,9

1Department and Graduate Institute of Veterinary Medicine, School of Veterinary Medicine National Taiwan University, Taipei 10617
2School of Medicine, Kaohsiung Medical University, Kaohsiung 80708
3Division of Neurosurgery, Department of Surgery, Changhua Christian Hospital, Changhua 50006
4Department of Nursing, College of Medicine and Nursing, Hungkuang University, Taichung 43302
5Department of Plastic and Reconstructive Surgery, China Medical University and China Medical University Hospital, Taichung 40447
6Graduate Institute of Immunology, China Medical University, Taichung 40402
7Center for Neuropsychiatry, China Medical University Hospital, Taichung 40402
8Division of Neurosurgery, Tungs’ Taichung Metroharbor Hospital, Taichung 43503
9Institute of Veterinary Clinical Science, School of Veterinary Medicine, National Taiwan University Taipei 10617, Taiwan, Republic of China

Abstract

Hydrocortisone is a growth hormone frequently used in the treatment of low back pain. Hydrocortisone treatment has an anti-inflammation effect, which also inactivates glucose transporter type 4 (GLUT4) by p38 mitogen-activated protein kinase (MAPK) inhibition. Translocation of GLUT4 regulates body glucose homeostasis and muscle repair and is induced by insulin. In this study, 56 SD rats were divided into seven groups, and were treated with insulin or hydrocortisone in sedentary or exercise training groups. The muscle proteins and biochemical blood parameters were analyzed after 7 days of treatments. The results showed that the serum glucose increased in hydrocortisone treatment accompanied by GLUT4 inactivation in both the sedentary and exercise training rats. In the exercise training groups, GLUT4 was redistributed on the plasma membrane on co-treatment with insulin and hydrocortisone through Akt phosphorylation. Insulin treatment exerted a compensatory feedback effect on the GLUT4 translocation on hydrocortisone co-treatment, which was the cause of GLUT4 inactivation.

Key Words: Akt phosphorylation, GLUT4, hydrocortisone, insulin, skeletal muscle

Introduction

Some patients suffering from chronic low back pain after strenuous exercise, and hydrocortisone injection usually provides instant cure affection (4, 18, 29). There were several regimens using hydrocortisone...
for anti-inflammation or pain relief after surgery (7, 11, 17, 26). However, the optimal treatments or adjuvant therapies seem unavailable, and relatively little is known about the involved molecular mechanisms of the hydrocortisone action. Such patients continue to suffer from chronic low back pain in a vicious circle of the same etiology.

The use of hormone preparations, such as anabolic steroids, growth hormone and insulin, appear to be increasing and their use is widespread in sport, herbal medicines and nutritional supplements (2, 8, 13). Despite some adverse effects due to overdose or abuse, it is currently legal and its use by athletes is not considered as doping (6). The so-called “natural products”, “health foods”, “dietary supplements” and the like often contain such hormone preparations, which have been clarified by broad-based investigations of the international nutritional supplement market (10). It is now critical to understand the mechanisms of the action of muscle remodeling and pain relief by hydrocortisone to facilitate proper use of these nutritional supplements.

Hydrocortisone is a steroid hormone produced by the adrenal gland in the zona fasciculate and the main function of hydrocortisone is pro-inflammation cytokine release inhibition (5). Particularly, the hydrocortisone can inhibit p38 mitogen-activated protein kinase (MAPK) activation in cytokine simulation (28). Moreover, a study pointed out that 2 mM hydrocortisone could improve the charcoal-treated fetal calf serum and restored 75% of the lost mitogenicity in Schwann cells (21). This may help the synaptic coupling cells and reduces pain.

The skeletal muscle cells are remodeled through glucose transporter type 4 (GLUT4) activation and translocation to the plasma membrane (16). Although AMPK can cause GLUT4 translocation, p38 MAPK is a stronger inducer (20, 27). Hydrocortisone may block skeletal muscle proliferation by its p38 MAPK suppression effect. Recently, insulin was reported to possess the GLUT4 directive activation inducer through AKT rather than the AMPK or p38 MAPK pathway (22, 31).

In order to define the benefits of insulin co-treatment in hydrocortisone treatment, the exercise-training animal model was used in this work. p38 MAPK and GLUT4 translocation were shown to be recovered by insulin in hydrocortisone treatments. The results might explain the benefit of insulin and hydrocortisone co-treatment in therapeutic effects of clinical muscle pain.

Materials and Methods

Animal Model

There were 56 SD rats (250 g, aged 6 weeks) purchased from BioLASCO Taiwan Co., Ltd., which were divided into 7 groups (n = 8 each). Groups designed were set as control, hydrocortisone treatment only (2 mg/kg/day), insulin treatment only (2 IU/kg/day), exercise training only, exercise training co-treated with hydrocortisone (2 mg/kg/day), exercise training co-treated with insulin (2 IU/kg/day), exercise training co-treated with insulin (2 IU/kg/day) and hydrocortisone (2 mg/kg/day). All the rats were treated once as indicated and were then fasted overnight for blood glucose administration before sacrificed. The exercise training was 30 min swimming in 25°C warm water. Insulin glargine (3.64 mg/ml, 100 IU/ml) was purchased from Sanofi-Aventis (Paris, France), and was diluted to 2 IU/ml in phosphate-buffered saline (PBS) pH 4.0, as the stock solution. Hydrocortisone was purchased from Sigma-Aldrich (Missouri, USA), and was dissolved in PBS at 2 mg/ml as the stock solution. Hydrocortisone and insulin treatments were applied by subcutaneously injection into the rats for 4 h before the exercise training or sedentary sessions. All procedures involving laboratory animal use were in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chung-Shan Medical University Experimental Animal Center (IACUC approval No. 844) for the care and use of laboratory animals.

Biochemical Blood Parameters

Blood samples were taken from the jugular veins then centrifuged at 2500 g (4°C, 20 min) for serum isolation. Assessments were analyzed on a Beckman Synchron CX9 clinical system, and serum parameters included alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), urea, uric acid (UA) and glucose (GLU).

Protein Extraction

All skeletal muscle tissue extracts of 8 rats in each group were obtained by homogenizing in a lysis buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA) at a ratio of 100 mg tissue/ml lysis buffer. The homogenates were placed on ice and then centrifuged at 13,000 rpm for 40 min. The supernatants were collected and stored at -80°C for further experiments. For GLUT4 translocation analysis, the protein samples used were homogenized in a buffer with 10 mM Tris-HCl, 1 mM EDTA, 250 mM sucrose. All samples were then subjected to differential centrifugations and store at -80°C until analysis.

Immunoblotting

Protein concentrations of skeletal muscle tissue
extracts were determined by the Lowry protein assay. Protein samples were separated in a 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a constant voltage of 75 V for 120 min. Proteins were then transferred to Hybond-C membranes (GE healthcare UK Ltd., Buckinghamshire, UK) using 50 V for 3 h. PVDF membranes were incubated in 3% bovine serum albumin (BSA) in TBS buffer. Primary antibodies, all purchased from Cell Signaling, MD, USA, including α-tubulin (3873S), Akt (9272S), p-Akt (9271S), GLUT4 (2213S), p-p38 (9211S), and p38 (9212S), were added into the membranes for recognizing the fitted proteins. Finally, horseradish peroxidase (HRP)-labeled antibodies were used and images were taken with Fujiﬁlm LAS-3000 (GE healthcare UK limited). The band intensities were quantiﬁed by scanning and processed using the Image J v1.44 edition.

**Cell Culture**

Mouse myoblast C2C12 cells were purchased from ATCC (Manassas, VA, USA) and were cultured in Dulbecco’s Modiﬁed Eagle’s Medium (DMEM) with 10% fetal bovine serum, 4.5 g/L glucose, in 5% CO₂ atmosphere at 37°C. After 2 days of incubation, the cultured medium was changed to DMEM with 2% horse serum for C2C12 myotube differentiation.

**ELISA Assay**

The mouse glucose transporter 4 ELISA kit (MyBioSource, Inc., CA, USA) was used to determine the GLUT4 activity of the cells. After drug treatments for 15 min, the cells were harvested and incubated together with GLUT4-HP conjugate in pre-coated plates. After 1 h incubation, the wells were decanted and washed 5 times with a wash solution. The wells were then incubated with the substrate for the HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. A stop solution was next added to stop the reaction. The intensity of color was measured spectrophotometrically at 450 nm in a microplate reader.

**Statistical Analysis**

All the results were obtained from 8 rats in each experimental group and are presented as group mean ± SD in tables or in the text. One-way analysis of variance was used to indicate an overall statistical signiﬁcance among the means of the seven experimental groups. For paired samples, the Student’s t-test was applied. A P-value of less than 0.05 was considered as signiﬁcant. Statistical analyses were performed using the SigmaPlot 11.0 edition.

**Results**

Data of the biochemical blood parameters analysis are shown in Table 1. The hydrocortisone treatment only in the sedentary group of rats did not affect the parameters other than the glucose concentration, which increased to 154.59 ± 9.62 mg/dl from 119.67 ± 5.66 mg/dl (control). In the insulin treatment only group, glucose levels decreased to 99.81 ± 6.72 mg/dl. Particularly, the serum glucose in the exercise training control group was slightly less than that of the sedentary control group. Although insulin treatment could reduce the serum glucose level, exercise training combined with insulin treatment could enhance more serum glucose reduction. There were no specific differences between the serum glucose levels in the exercise training co-treated with insulin and hydrocortisone group and the sedentary control group.

Moreover, the CK expression levels in all the exercise training groups were slightly higher than those in the sedentary groups (Table 1). But the CK expression levels were also slightly decreased in exercise training co-treated with hydrocortisone or co-treated with both hydrocortisone and insulin. The highest CK expression levels appeared in the sera of exercise training combined with insulin treatment group of rats.

**Table 1. The serum biochemistry markers analysis**

<table>
<thead>
<tr>
<th></th>
<th>Sedentary</th>
<th>Exercise</th>
<th>Hydrocortisone +</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hydrocortisone</td>
<td>Insulin</td>
</tr>
<tr>
<td></td>
<td>Hydrocortisone</td>
<td>Insulin</td>
<td>Hydrocortisone +</td>
</tr>
<tr>
<td>CK</td>
<td>933.74 ± 97.86</td>
<td>868.01 ± 113.50</td>
<td>921.39 ± 107.44</td>
</tr>
<tr>
<td>ALT</td>
<td>26.73 ± 2.30</td>
<td>25.64 ± 2.15</td>
<td>24.95 ± 2.54</td>
</tr>
<tr>
<td>AST</td>
<td>127.32 ± 9.76</td>
<td>126.55 ± 10.20</td>
<td>125.19 ± 9.99</td>
</tr>
<tr>
<td>Urea</td>
<td>23.17 ± 1.99</td>
<td>23.45 ± 2.03</td>
<td>23.21 ± 2.01</td>
</tr>
<tr>
<td>UA</td>
<td>0.70 ± 0.05</td>
<td>0.70 ± 0.04</td>
<td>0.71 ± 0.05</td>
</tr>
<tr>
<td>GLU</td>
<td>119.67 ± 5.66</td>
<td>154.59 ± 9.62**</td>
<td>99.81 ± 6.72*</td>
</tr>
</tbody>
</table>

All plasma samples (n = 8) were analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), urea, uric acid (UA), and glucose (GLU). *P < 0.05, **P < 0.01, ***P < 0.001, compared with the sedentary control group; #P < 0.05, ##P < 0.01, compared with the exercise control group.
Hydrocortisone upstream activators are Akt and p38 MAPK. These active kinase proteins can be measured by the ratio between phosphorylated and native unphosphorylated protein forms. The p-Akt expression levels in the sedentary groups were similar (Fig. 1). p-Akt expression was enhanced in the exercise training control group, and could be reduced after hydrocortisone treatment. Additionally, insulin treatment reversed the p-Akt expression ratio on co-treatment with hydrocortisone, or enhanced p-Akt expression in exercise training. Moreover, the p-p38 expression levels were inhibited in each of the hydrocortisone treatment groups, and exercise training or insulin treatment still failed to overcome the p38 MAPK inhibitory effects.

Fig. 1. Expression analysis of GLUT4 translocation upstream kinases. (A) After 7 days of the indicated treatments, the active portions of Akt and p38 MAPK were analyzed by western immunoblotting assay. The folds of protein expression were normalized to that of α-tubulin. The ratio between phosphorylated kinases (active form) and native form are shown in (B) and (C). *P < 0.05, **P < 0.01, compared with the respective control group.
GLUT4 activation can be defined by its plasma membrane translocation. GLUT4 expression in the cytosol and the plasma membrane of each treatment group is shown in Fig. 2. In the sedentary groups, only the insulin treatment could increase the GLUT4 translocation to the plasma membrane. In the exercise groups, insulin treatment enhanced the GLUT4 translocation ratio from 3.16 fold up to 4.87 fold. Although the hydrocortisone could inhibit the GLUT4 translocation in the exercise training group, in which the ratio of GLUT4 translocation was 0.06. But GLUT4 translocation was compensatively reversed by insulin treatment, and the ratio of GLUT4 translocation was 4.51.

Additionally, the C2C12 myotubes were treated with insulin (10 mU/ml) and hydrocortisone (100 nM) for 15 min. The p-p38 and p-Akt expression levels were not enhanced on hydrocortisone treatment, but p-p38 and p-Akt were increased in the insulin treatment groups (Fig. 3A). These results were similar to the animal model results in this research. Furthermore,
Hydrocortisone and Insulin Co-treatments in Skeletal Muscle

the p38 and Akt kinase activation were blocked by the p38 and Akt1/2 kinase inhibitor pretreatment in C2C12 myotubes (Figs. 3B & 3C). The GLUT4 activity of C2C12 myotubes was analyzed by the same indicated treatments. The hydrocortisone treatment inhibited the GLUT4 translocation activity and insulin enhanced it (Fig. 4). Further, the GLUT4 translocation activities enhanced by insulin were blocked by both p38 and Akt1/2 kinase inhibitor pretreatments in C2C12 myotubes.

Discussion

An exercise training-induced muscle damage is regulated to oxidative stress in impaired insulin signaling pathway and releases the CK into serum; hence, CK elevations indicate muscle damage and inflammation (1, 23). Hydrocortisone is an efficient anti-inflammation drug owing to its TGF-β inhibitory properties, and is applied in muscle pain treatments and muscle recovery (14, 15). In this research, the CK expression levels were found to be high in exercise training groups, and hydrocortisone treatment, indeed, partially decreased CK elevations in the serum (Table 1).
GLUT4 Activity Folds

Fig. 4. Analysis of GLUT4 translocation expression in C2C12 myotubes. After 6 h of p38 inhibitor (SB203580, 10 nM) and Akt1/2 kinase inhibitor (50 nM) treatment, insulin (10 mU/ml) was cotreated with the C2C12 myotubes for an additional 15 min. The GLUT4 translocation activities were analyzed by ELISA assays compared to the control and insulin (10 mU/ml) or hydrocortisone (100 nM) only treatment for 15 min. ***p < 0.01, **p < 0.001, compared with the respective control group.

When skeletal muscles contract, AMPK is activated, with a concurrent increase in free AMP concentration (12, 25). The principal glucose transporter protein GLUT4 plays a key role in regulating whole-body glucose homeostasis (11). Moreover, p38 MAPK is a strong inducer of GLUT4 and hydrocortisone can act as an inhibitor of p38 MAPK (20, 28), which causes a problem in hydrocortisone application in muscle repairing processes. Here, the GLUT4 translocation ratio was shown to increase in the muscles of the exercise training groups of rats, and this effect was blocked by hydrocortisone treatment (Fig. 2). In fact p38 MAPK expression was all inhibited in the muscle of each hydrocortisone rat.

Furthermore, an important role for Akt in the stimulation of glucose transport through GLUT4 translocation by insulin in muscle has been reported (19, 30). The insulin signaling pathway is triggered by the activation of the insulin receptor (IR) and downstream tyrosine kinase, leading to tyrosine phosphorylation of insulin receptor substrate proteins (IRS) and recruitment of PI3K. Subsequently, the phosphorylated PI3K activates the Akt and further induces GLUT4 translocation to the membrane (3).

In this work, the ratios of phosphorylated Akt were increased in each insulin treatment group (Fig. 1), and downstream GLUT4 translocation was not inhibited. Interestingly, the original GLUT4 translocation inhibition by hydrocortisone treatment was also reversed to the activated form (24). The GLUT4 translocation activities enhanced by insulin were blocked by both p38 and Akt1/2 kinase inhibitor pretreatment in C2C12 myotubes (Fig. 4). These results suggested that activation of the p38 and Akt kinase plays the key role in insulin-dependent GLUT4 translocation activity in C2C12 myotubes. This experimental evidence supported that GLUT4 translocation was controlled by dual Akt and p38 MAPK pathways, and Akt is a dominator although p38 MAPK is still a strong inducer of GLUT4. Insulin co-treatment has a compensatory feedback effect on the GLUT4 translocation on hydrocortisone treatment that causes GLUT4 inactivation.

References


