Investigation of the pronounced erythropoietin-induced reduction in hyperglycemia in type 1-like diabetic rats

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Abstract. Erythropoietin (EPO) is known to stimulate erythropoiesis after binding with its specific receptor. In clinics, EPO is widely used in hemodialyzed patients with diabetes. However, changes in the expression of the erythropoietin receptor (EPOR) under diabetic conditions are still unclear. Therefore, we investigated EPOR expression both in vivo and in vitro. Streptozotocin-induced type 1-like diabetic rats (STZ rats) were used to evaluate the blood glucose-lowering effects of EPO. The expression and activity of the transducer and activator of transcription 3 (STAT3), the potential signaling molecule, was investigated in cultured rat skeletal myoblast (L6) cells incubated in high-glucose (HG) medium to mimic the in vivo changes. The EPO-induced reduction in hyperglycemia was more pronounced in diabetic rats. The increased EPOR expression in the soleus muscle of diabetic rats was reversed by the reduction in hyperglycemia. Glucose uptake was also increased in high-glucose (HG)-treated L6 cells. Western blotting results indicated that the EPO-induced hyperglycemic activity was enhanced mainly through an increase in EPOR expression. Increased EPOR expression was associated with the enhanced nuclear expression of STAT3 in HG-exposed L6 cells. In addition, treatment with siRNA specific to STAT3 reversed the increased expression of EPOR observed in these cells. Treatment with Stattic at a dose sufficient to inhibit STAT3 reduced the expression level of EPOR in STZ rats. In conclusion, the increased expression of EPOR by hyperglycemia is mainly associated with an augmented expression of nuclear STAT3, which was identified both in vivo and in vitro in the present study.

Key words: Erythropoietin receptor, Transducer and activator of transcription 3, Streptozotocin-induced type 1-like diabetic rats, Hyperglycemia

THE HEMATOPOIETIC GROWTH FACTOR ERYTHROPOIETIN (EPO) is produced in the kidney in response to hypoxia and stimulates erythropoiesis in bone marrow [1]. Recombinant human EPO (rHuEPO) is an effective treatment for various types of anemia, including anemia associated with renal failure [2] and with cancer-related diseases [3]. The major function of EPO is mediated by its specific cell-surface receptor, erythropoietin receptor (EPOR). EPORs have been found in multiple tissues, suggesting a role for EPO in non-

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Abbreviations: EPO, erythropoietin; rHuEPO, Recombinant human EPO; EPOR, erythropoietin receptor; L6, rat skeletal myoblast; STZ rats, Streptozotocin-induced type 1-like diabetic rats; STAT3, transducer and activator of transcription 3; HG, high glucose; 2-NBDG, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose; PBS, phosphate buffer solution; RIPA buffer, radio-immuno-precipitation assay buffer; HMBS, hydroxymethylbilane synthase
hematopoietic tissues, including the brain [4], retina, kidneys, smooth muscle cells, myoblasts, vascular endothelia, and heart, and in other cell types [5, 6]. EPORs in neuronal cells are induced by hypoxia, which results in increased EPO sensitivity [7]. After binding to EPOR, EPO facilitates several activities in the cardiovascular system. In cardiac cells, EPO protects cardiomyocytes against ischemic injury without an increase in hematocrit, demonstrating that EPO can directly protect the ischemic and infarcted heart [8].

Hyperglycemia is a central factor in the induction of diabetic complications, particularly in nephropathy [9]. It has been suggested that establishing glycemic control early reduces the incidence of diabetic nephropathy [10]. Hyperglycemic damage to mesangial cells is implicated in the development of diabetic nephropathy [11]. The positive effect of rHuEPO treatment on glucose homeostasis in hemodialyzed patients established the need for the interplay between EPO and glucose in the absence of clinical problems to be critically explored [12].

EPO is widely used in diabetic patients with chronic kidney disease [13]. The positive effect of EPO on glucose homeostasis during hemodialysis has been reported in clinics [12]. In addition, the effects of EPO on lipid metabolism and the hypoglycemic response have been observed [14]. EPO-induced reductions in hyperglycemia have been indicated in a diabetic mouse model and in ob/ob mice [15]. In high-fat-fed and low-dose streptozotocin-induced experimental diabetic rats, EPO modulated glucose metabolism, decreased fasting blood glucose levels, and improved glucose tolerance and insulin sensitivity [16]. We also demonstrated that EPO ameliorated hyperglycemia in rats with type 1 diabetes [17]. However, the differences in the response to EPO between normal and diabetic animals remain obscure. In the present study, we investigated the effect of hyperglycemia on EPO-induced responses using type 1-like diabetic rats with severely diminished circulating insulin levels [18].

Hyperglycemia-induced STAT3 signaling pathway activation has been shown to be an integral part of tissue damage, including myocardial infarction, oxidative damage, liver fibrosis, pulmonary fibrosis and renal fibrosis [19]. Furthermore, the activation of STAT3 may contribute to the transcriptional regulation of genes [20]. We previously demonstrated that the increase in STAT3 expression caused by hyperglycemia is involved in lung damage in diabetic rats [21]. Therefore, the role of STAT3 in the altered EPO response was also investigated in the present study.

Materials and Methods

Animal model

Six-week-old male Wistar rats were obtained from the Animal Center of the National Cheng Kung University Medical College. Diabetes was induced in the rats by intravenously (i.v.) injecting streptozotocin (65 mg/kg) into fasting rats as previously described [22]. In accordance with a previous study [23], the animals were considered diabetic if they had plasma glucose concentrations over 350 mg/dL. The plasma insulin levels in the STZ-diabetic rats decreased to less than 8% of that measured in the normal rats. All experiments were performed 9 weeks after diabetes induction. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Drug treatment

To investigate the effect of EPO on the plasma glucose levels of diabetic rats, a stock solution of recombinant human erythropoietin (Roche, Mannheim, Germany) was diluted in 9% normal saline. EPO (100 IU/kg) was administered to the STZ rats by intravenous injection once a day for 7 days (n = 6 per group) [17]. At the end of the experiment, blood samples were obtained from the tail veins of rats under anesthesia with sodium pentobarbital (35 mg/kg, ip). The concentration of plasma glucose in each sample was then estimated by an autoanalyzer (Quik-Lab, USA) and run in duplicate. The difference in the response to the tested compound between groups was also compared for hypoglycemic activity (%), which was calculated as the percent reduction from the initial glucose value according to the formula (Gi - Gt)/Gi × 100%, where Gi is the initial glucose concentration and Gt is the plasma glucose concentration after treatment with the tested compound, as described in our previous report [18].

Cell cultures

Rat skeletal myoblast (L6) cells (ATCC No. CRL1458) were obtained from the Culture Collection and Research Center of the Food Industry Institute (Hsin-Chiu City, Taiwan) and were maintained in Dulbecco’s modified Eagle’s medium (Gibco-BRL, USA) supplemented with 10% fetal bovine serum and antibiotics. After the cells reached approximately 60% confluent...
ence, they were incubated in serum-free medium with varying working concentrations of D-glucose (5.5, 10 or 20 mM) for 0, 12 or 24 hours. L6 cells exposed to 5.5 mM D-glucose were used as the control. To rule out an osmolarity effect, we added 14.5 mM mannitol to the control cultures.

**Uptake of 2-NBDG into L6 Cells**

The glucose uptake test was performed according to a previous report with some modifications, using 2-((N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) as a fluorescence indicator [24]. The L6 cells were cultured in a 10-cm dish for 48 h, and the concentration of cells in each assay was 1 × 10^6 cells/mL. The medium was removed, and the cells were washed gently with phosphate-buffered saline (PBS). Cells were detached from the dish by using a trypsin treatment, suspended in PBS with 0.2 mM 2-NBDG and the compound to be tested at the indicated concentration, and then incubated in a 37°C water bath for 60 min in the dark. The cells were centrifuged (4°C, 5000 × g, 10 min), and the supernatant was discarded. The pellet was washed three times with cold PBS and cooled on ice. The pellet was then suspended in 1 mL of PBS. The fluorescence intensity in the cell suspension was evaluated using a fluorescence spectrofluorometer (Hitachi F-2000, Tokyo, Japan) with excitation and emission wavelengths of 488 and 520 nm, respectively. The intensity of the fluorescence reflected the uptake of 2-NBDG into the cells [25].

**Small Interfering RNA (siRNA)**

The duplex RNA oligonucleotides for rat STAT3 (siGENOME SMARTpool™) were prepared by Thermo Fisher Scientific Inc. L6 cells were transfected with 40 pmol of either STAT3-specific siRNAs (siSTAT3) or scrambled siRNA (Sc) according to the manufacturer’s protocols. The cells were then incubated for 48 hours after transfection before being used for further investigation.

**Nuclear protein extraction**

For nuclear protein extraction, as described in our previous report [25], a CNMCS Compartamental Protein Extraction Kit (BioChain Institute, Inc., Hayward, CA, USA) was used in the present study. Briefly, rat muscle homogenates and cells were collected, ice-cold lysis buffer (2 mL per 20 million cells) was added, and the mixture was rotated at 4°C for 20 min. The mixture was then placed in a syringe attached to a 26.5 to 30-gauge needle. The needle tip was removed by bending the needle several times, leaving only the needle base on the syringe. The cell mixture was passed through the needle base 50–90 times to disrupt the cell membranes and to release the nuclei from the cells. The degree of cell membrane disruption and the release of nuclei were monitored with a microscope. The mixture was then centrifuged at 15,000 × g at 4°C for 20 min. The supernatant, which contained cytoplasmic proteins, was removed and saved in a separate tube. The pellet was resuspended in ice-cold wash buffer (4 mL per 20 million cells), and the suspension was rotated at 4°C for 5 min, followed by centrifugation at 15,000 × g at 4°C for 20 min. The supernatant was then removed, and ice-cold nuclear extraction buffer (1 mL per 20 million cells) was added to the pellet. After rotating at 4°C for 20 min, the suspension was centrifuged at 15,000 × g at 4°C for 20 min. The supernatant, which contained nuclear proteins, was removed and saved for further experimentation.

**Western blotting analysis**

As in our previous report [25], protein was extracted from rat soleus muscle homogenates and cell lysates using ice-cold radio-immuno-precipitation assay (RIPA) buffer supplemented with phosphatase and protease inhibitors (50 mmol/L sodium vanadate, 0.5 mM phenylmethylsulphonyl fluoride, 2 mg/mL aprotinin, and 0.5 mg/mL leupeptin). Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total protein (30 μg) was separated with SDS/polyacrylamide gel electrophoresis (10% acrylamide gel) using a Bio-Rad Mini-Protean II system. The protein was transferred to polyvinyldiene difluoride membranes (Pierce, Rockford, IL, USA) with a Bio-Rad Trans-Blot system. Membranes were blocked with 5% non-fat milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) and incubated for two hours. The membranes were then washed in PBS-T and hybridized with primary antibodies, which were diluted to a suitable concentration in PBS-T for 16 hours. Antibodies specific for STAT3 and EPOR (1:1,000) were used. After incubation with secondary antibodies, detection of the antigen–antibody complex was performed using an ECL kit (Amersham Biosciences, UK). Immunoblot densities of the bands for STAT3 (88 kDa), EPOR (55 kDa), and β-actin (43 kDa) were quantified using a laser densitometer.
Real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from muscle homogenates and cell lysates with TRIzol reagent (Carlsbad, CA, USA). The web-based assay-design software from the Universal Probe Library Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp) was used to design TaqMan primer pairs and to select appropriate hybridization probes. All PCR experiments were performed using a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany). Reactions were performed in a total volume of 20 μL consisting of 13.4 μL of PCR buffer, 0.2 μL of each Universal Probe Library probe (10 mmol/L), 0.2 μL of each primer (20 μmol/L) (Table 1), 4 μL of LightCycler TaqMan Master mix (Roche Diagnostics GmbH) and 2 μL of template cDNA. The thermal cycling conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 45 cycles at 94°C for 10 s, 60°C for 20 s, and 72°C for 1 s. The crossing point for each amplification curve was determined using the second derivative maximum method. The concentration of each gene was calculated in reference to the respective standard curve with the aid of the LightCycler software. Relative gene expression was expressed as the ratio of the concentration of the target gene to that of the housekeeping gene hydroxymethylbilane synthase (HMBS), as in our previous report [25].

Statistical analysis

The results are shown as the mean ± SEM of each group with the indicated sample number (n). One-way analysis of variance (ANOVA) was performed, which was followed by a Tukey’s post hoc test. The datasets of two groups were analyzed with an independent t-test. A p-value of 0.05 or less was considered significant.

Results

EPO-induced reduction in hyperglycemia is augmented and EPOR expression is increased in diabetic rats

Nine weeks following the induction of diabetes, serum glucose levels in the STZ-treated rats were markedly (p < 0.05) higher than in the age-matched normal rats (Table 2). EPO-induced a dose-dependent decrease in the blood glucose concentration in rats, as shown in Fig. 1A, and the hypoglycemic response to EPO was more pronounced in the diabetic rats. Blood glucose was reduced from 473.11 ± 17.07 mg/dL to 390.97 ± 17.83 mg/dL in the diabetic rats and from 96.22 ± 16.42 mg/dL to 77.51 ± 4.93 mg/dL in the normal rats after treatment with the same dose of EPO (100 IU) for 120 min. Western blots revealed that EPOR expression (Fig. 1B) in the soleus muscle of the diabetic rats was increased compared to that of the normal control rats. After a 7-day treatment with insulin or phloridzin, the plasma glucose level was markedly reduced in the diabetic rats (Table 2). Insulin or phloridzin treatment, at a dose sufficient to correct hyperglycemia, reversed the EPOR expression in the skeletal muscle of diabetic rats (Fig. 1B).

Increased EPO-induced glucose uptake and EPOR expression in high-glucose-treated L6 cells

Changes in EPOR expression were further investigated in cultured L6 cells treated with high glucose (20 mM) for various time intervals (0, 6, 12 or 24 hours). EPOR expression in L6 cells was also markedly increased by high glucose in a time-dependent manner (Fig. 2A). The most striking time point was at 12 hours, which was then used for further analysis. In addition, changes in EPOR expression were investigated in cultured L6 cells by incubation with glucose at various concentrations for 12 hours in vitro. EPOR expression in L6 cells was significantly increased by higher glucose concentrations in a concentration-dependent manner (Fig. 2B). However, EPOR expression was not changed by mannitol at a concentration that produced an equivalent osmolarity to that produced by high glucose in L6 cells. The possibility that hyperosmolarity mediated this change in EPOR expression can thus be excluded. The most effective high-glucose condition (20 mM for 12 hours) was used for real-time PCR experiments. The mRNA expression level of EPOR in L6 cells was also increased by high glucose (Fig. 2C). Moreover, using the 2-NBDG method, glucose uptake was significantly increased in high-glucose-treated L6 cells.
increased in a concentration-dependent manner by EPO expression in high-glucose-treated L6 cells (Fig. 2D). Thus, high-glucose-induced EPOR expression was identified in L6 cells, which is similar to the observations of EPOR expression diabetic rats (Fig. 1).

**Increase in the nuclear STAT3 expression level in L6 cells by high glucose**

We focused on the changes in nuclear STAT3 levels in cultured L6 cells incubated with high glucose at various time points (0, 5, 10 and 20 hours). Nuclear STAT3 in L6 cells was also increased by high glucose in a time-dependent manner. Interestingly, the most significant time point was at 10 hours (Fig. 3A), which was earlier than that observed for EPOR expression. Additionally, nuclear STAT3 in L6 cells was markedly increased by high glucose in a concentration-dependent manner after a 10-hour incubation (Fig. 3B). The increased expression of nuclear STAT3 by high glucose in L6 cells was similar to the dose-dependent increase in EPOR expression (Fig. 2). However, the nuclear STAT3 expression level was not changed by mannitol in L6 cells, suggesting that the changes in nuclear STAT3 are unlikely to be mediated by hyperosmolarity.

**Effects of STAT3 siRNA on the high-glucose-induced expression of EPOR in L6 cells**

L6 cells were transfected with either siRNA specific to STAT3 (siSTAT3) or control scrambled siRNA (Sc) for 48 hours in accordance with a previous report [7]; STAT3 expression was substantially reduced in L6 cells, thus confirming the transfection efficiency (Fig. 4A). In addition, the high-glucose-induced changes in EPOR, protein and mRNA expression levels were reversed by silencing STAT3 in L6 cells (Fig. 4B, C).

**The effect of a STAT3-specific inhibitor (Stattic) on the expression of EPOR**

Changes in EPOR expression (Fig. 5A) and EPO-induced glucose uptake (Fig. 5B) were both reversed by
Stattic in the high-glucose-treated L6 cells. Stattic was then used at a dose sufficient to inhibit STAT3, as described previously in STZ rats [26]. The increased expression of EPOR and the EPO-induced reduction in hyperglycemia in STZ rats were also reversed by Stattic (Fig. 6) at a dose sufficient to inhibit STAT3 but insufficient to alter hyperglycemia (Table 3). This result is consistent with the results observed in L6 cells, which demonstrates the role of nuclear STAT3 in the increased expression of EPOR in STZ rats.

**Discussion**

In the present study, we found that the enhanced EPO-induced reduction in hyperglycemia was associated with increased EPOR expression in the skeletal muscle of type 1-like diabetic rats. Insulin and phloridzin effectively lowers blood glucose in diabetic animals through different mechanisms: insulin decreases blood glucose via receptor-coupled signaling [27], but phloridzin acts as an inhibitor of renal tubular glucose reabsorption [28]. A 7-day treatment with either insulin or phloridzin corrected hyperglycemia and reversed the EPOR expression in STZ-induced diabetic rats. These findings indicate that

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**Fig. 2** Changes in EPOR expression and EPO-induced glucose uptake in L6 cells after exposure to high glucose. We investigated the changes in EPOR expression in cultured L6 cells *in vitro* after incubation either with glucose for the indicated timepoints (0, 6, 12 and 24 hours) (A) or with various glucose concentrations (0, 10 and 20 mM) (B). The protein expression levels, using EPOR expression normalized to that of β-actin, are indicated in the lower panel of each column. The related mRNA expression, as detected using real-time PCR and quantified using the ratio of EPOR to HMBS, is represented (C). Glucose uptake was estimated using 2-NBDG with various concentrations of glucose (D). Data are shown as the ± SEM (n = 6 per group); *p < 0.05, **p < 0.01 and ***p < 0.001 compared to the control (no addition of glucose for 0 hours).
hyperglycemia induced an increase in EPOR expression, which has not been studied. In addition, we treated a rat skeletal myoblast cell line, L6, with high glucose to mimic diabetes and confirmed the increased EPOR expression along with enhanced glucose uptake by means of 2-NBDG uptake experiments. Interestingly, the nuclear STAT3 expression level was also upregulated similar to the increased EPOR expression. Moreover, silencing of STAT3 reversed the increased expression of EPOR that was induced by high glucose in the L6 cells. The STAT3-specific inhibitor (Stattic) also reversed the change in EPOR expression induced by hyperglycemia both in vitro and in vivo. As evidenced by our findings, hyperglycemia may increase the nuclear STAT3 expression level and consequently increase EPOR expression, resulting in a more pronounced EPO-induced reduction in hyperglycemia in diabetic animals. However, no difference in the plasma glucose concentration has been indicated between patients receiving EPO or placebo treatment in a clinical study [29]. Many factors may be responsible for the different results, but more evidence from clinical trials will be helpful in the future.

The reduction in blood glucose by EPO is thought to correlate with an increase in cellular glucose uptake [30]. In the present study, we found that the EPO-induced reduction in hyperglycemia was more pronounced in type 1-like diabetic rats than in normal rats. This result was also confirmed in cultured L6 cells using high-glucose medium. The pharmacological treatment of hyperglycemia in rats reversed the augmented EPO-induced reduction in hyperglycemia. It seems possible that hyperglycemia may increase the response to EPO both in vivo and in vitro.

The primary function of EPO is mediated by the specific cell-surface receptor erythropoietin receptor (EPOR) [31]. The expression of EPOR in non-erythroid tissues or cells, such as the brain [32], retina [33], heart [34], kidneys [35], smooth muscle cells [36], myoblasts [5] and vascular endothelia [37], has been associated with the discovery of various biological functions regulated by endogenous EPO signaling in non-hematopoietic tissues and with the ability of exogenous EPO to modulate organ function and the cellular response to diverse types of injury [38]. EPOR has been reported to be upregulated during harmful stress [39]. In contrast to EPO, the expression of EPOR is strongly stimulated by proinflammatory cytokines, particularly under conditions of metabolic stress [40]. In the present study, we identified an increase in the expression of EPOR in type 1-like diabetic rats, and this finding was reversed by the correction of hyperglycemia using insulin or phloridzin. Furthermore, the expression of EPOR was increased by high glucose in a time- and dose-dependent manner in L6 cells. Therefore, the increased expression of EPOR in
Hyperglycemia is related to the heightened response of EPO-induced actions in diabetes. STAT3 is a cytoplasmic transcription factor that transmits extracellular signals to the nucleus [41]. Activated STAT3 in the nucleus binds to specific DNA promoter sequences and regulates gene expression [42]. Recent studies have suggested that hyperglycemia increases STAT3 activation and thereby contributes to the pathophysiology of tissue injury [43]. Our data also showed a time- and dose-dependent increase in nuclear STAT3 expression in L6 cells incubated in high glucose. The increase in nuclear STAT3 by high-glucose treatment was produced at an earlier timepoint than the augmented EPOR expression. The dose-dependent change in nuclear STAT3 expression was associated with the increase in EPOR expression. Furthermore, silencing STAT3 in L6 cells reversed the increase in EPOR expression induced by high glucose. The pharmacological inhibitor (Stattic) specific to STAT3 also reversed the increase in EPOR expression both in vitro and in vivo without altering the blood glucose concentration. Therefore, STAT3 may be involved in the increase in EPOR expression. The specificity of Stattic has been challenged [44]. However, Stattic has been used as a specific STAT3 inhibitor in numerous studies [45, 46]. Furthermore, our siRNA-knockdown experiments support our hypothesis.

Glucose homeostasis is maintained by many factors, of which glucose uptake into skeletal muscle is a critical component [47]. Thus, we focused on the changes in skeletal muscle in the present study. Further studies...
should attempt to reproduce our findings in other insulin-sensitive tissues, such as liver or adipose tissue.

In summary, we suggest that hyperglycemia may increase both nuclear STAT3 and EPOR expression to enhance the EPO-induced reduction in hyperglycemia in diabetic rats. Our findings highlight a potential mecha-

**Table 3** Changes of blood glucose levels in streptozotocin-induced diabetic rats (STZ) receiving the repeated intravenous injection of stattic at 2 mg/kg daily for 7 days

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<td><strong>After</strong></td>
<td>402.5 ± 3.8</td>
<td>399.4 ± 5.7</td>
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Values (mean ± SE) were obtained from each group of six rats. The vehicle used to dissolve the testing drug was given at same volume.
nism for the response of EPO under hyperglycemic conditions and encourage the careful application of EPO treatment in clinics.

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EPO receptor increased in diabetes

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