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Original article

Dual effect of the heart-targeting cytokine cardiotrophin-1 on glucose transport in cardiomyocytes

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ABSTRACT

Cardiotrophin-1 (CT-1) is a heart-targeting cytokine that is increased in the metabolic syndrome due to overexpression in the adipocytes. The effects of CT-1 on cardiomyocyte substrate metabolism remain unknown. We therefore determined the effects of CT-1 on basal and stimulated glucose transport in cardiomyocytes exposed to a low dose (1 nM) or a high dose (10 nM). Dose–response curves for insulin showed that 1 nM CT-1 reduced insulin responsiveness, while 10 nM CT-1 increased insulin responsiveness. In either condition insulin sensitivity was unaffected. Similarly 1 nM CT-1 reduced the stimulation of glucose transport in response to metabolic stress, induced by the mitochondrial poison oligomycin, while 10 nM CT-1 increased this response. Reduction of stimulated glucose transport by 1 nM CT-1 was associated with overexpression of SOCS-3, a protein known to hinder proximal insulin signaling, and increased phosphorylation of STAT5. In cardiomyocytes exposed to 1 nM CT-1 there was also reduced phosphorylation of Akt and AS160 in response to insulin, and of AMPK in response to oligomycin. Insulin-stimulated glucose transport and signaling were restored by inhibition of STAT5 activity. On the other hand in cardiomyocytes exposed to 10 nM CT-1 there was increased phosphorylation of the AS160 and Akt in response to insulin. Most importantly, basal and oligomycin-stimulated phosphorylation of AMPK was markedly increased in cardiomyocytes exposed to 10 nM CT-1. The enhancement of basal and stimulated-glucose transport was abolished in cardiomyocytes treated with the calmodulin-dependent kinase II (CaMKII) inhibitor KN93, and so was AMPK phosphorylation. This suggests that activation of CaMKII mediates activation of AMPK by a high dose of CT-1 independently of metabolic stress. Our results point to a role for CT-1 in the regulation of myocardial glucose metabolism and implicate entirely separate mechanisms in the inhibitory or stimulatory effects of CT-1 on glucose transport at low or high concentrations respectively.

1. Introduction

The metabolic syndrome is a leading cause of mortality and morbidity in the Western hemisphere. A central feature of this syndrome is insulin resistance. Insulin resistance has been extensively investigated at the level of skeletal muscle, which, by virtue of its sheer bulk, is the premier metabolic target of insulin. However, insulin resistance of the heart muscle also occurs and could have more rapid dire consequences. Furthermore, insulin stimulation of myocardial glucose transport shares mechanisms with stimulation of myocardial glucose transport by metabolic stress [1], an important process for cardiomyocytes survival during ischemia and reperfusion episodes. The importance of stimulation of glucose metabolism for post-ischemic recovery is illustrated by the poor recovery of GLUT4-null [2] or AMPK-deficient [3] hearts, which are incapable of stimulation of glucose transport.

Insulin resistance is thought to be associated with chronic inflammatory state [4]. Major inflammatory markers that have been linked to the metabolic syndrome include TNF-α, adiponectin and IL-6 family members, among which cardiotrophin-1 (CT-1) [5]. Similarly to Leukemia Inhibitory Factor (LIF), CT-1 binds to a receptor complex comprising gp130 and the LIF receptor (LIFR) [6]. Binding of CT-1 to its receptor triggers activation of a wide array of signaling pathways [7]. *In vitro*, CT-1 has been found to promote not only cardiomyocytes hypertrophy but also resistance to apoptosis; nonetheless its effects on cardiomyocytes substrate metabolism remain unclear. By comparison, LIF, which shares the receptor complex with CT-1, induces insulin resistance after chronic exposure of cardiomyocytes [8].

The metabolic effects of CT-1 on non-myocardial tissues are controversial. Chronic exposure of adipocytes to CT-1 *in vitro* results in insulin resistance [9], yet CT-1 knock-out mice developed insulin resistance that could be reversed by administration of exogenous

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CT-1 [10]. Therefore potential effects of CT-1 on myocardial glucose metabolism could either participate in, if positive, or limit, if negative, its cardioprotective effects. Thus, we undertook the present study to determine the effects of CT-1 on basal and stimulated glucose transport in cardiomyocytes. For this purpose, we measured 2-deoxy-D-glucose (2-DG) uptake in adult rat cardiomyocytes left untreated or treated with CT-1. Furthermore, we investigated the effect of CT-1 on intracellular signaling in response to insulin and metabolic stress.

2. Materials and methods

2.1. Animals

We obtained male Sprague–Dawley rats (100–110 g) from Charles Rivers France (L'Arbresle, France). The ethical committee of the Geneva University School of Medicine and the Geneva State Veterinary Office approved the study protocol, which conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Adult rat cardiomyocytes culture

Adult rat cardiomyocytes were isolated as previously described [11] by retrograde perfusion of the hearts with collagenase (type II; Worthington) [12,13]. Cells were plated in M199 medium containing 5.5 mM glucose supplemented with 20 mM creatine, 100 μM cytosine-β-D-arabinofuranoside, and 20% fetal calf serum (FCS) in dishes previously coated with 0.1% gelatin for 4 h and incubated overnight with complete culture medium. Cardiotrophin-1 (R&D systems), STO 609 and KN-93 (Sigma-Aldrich), STAT 5 inhibitor (Calbiochem) were prepared according to the manufacturer's instructions and added to the medium at the time of plating (day 0). Subsequent analyses were performed on day 7. At this time point control cardiomyocytes display a well differentiated phenotype with stable insulin responsiveness [14].

2.3. Determination of 2-deoxy-D-glucose uptake

Glucose transport was estimated by measuring 2-deoxyglucose (2-DG) uptake, as previously described [11,15]. Briefly, cardiomyocytes were incubated in M199 containing 10 nM [2,6-^{3}H]-2-DG (GE Healthcare) (1–2 μCi/ml) and 5.5 mM cold glucose at 37 °C for 1 h, in the presence or absence of glucose transport agonists. Glucose transport agonists used were insulin (10^{-9}–10^{-7} M) or oligomycin (10^{-6} M) to induce metabolic stress. Glucose transport in response to hypoxia was measured in complete culture medium, including CT-1 at 1 or 10 nM. The 2-DG concentration was reduced to 1 nM. Hypoxia (1% O_2, 5% CO_2, balanced with N_2 at 37 °C) was maintained for 6 h in a hypoxic incubator (New Brunswick Scientific Innova CO-14); control incubations were conducted for the same time in 5% CO_2 at 37 °C in air.

2-DG uptake was stopped by adding 0.4 mM phloretin and the cells were washed three times with ice-cold PBS before lysis in 1 ml 0.1 M NaOH. Twenty-μl aliquots were taken for protein content determination and the remaining NaOH lysate assayed for radioactivity in a TriCARB 1900 TR liquid scintillation analyzer (Packard). Results are expressed as fold stimulation of 2-DG uptake with respect to control unstimulated cardiomyocytes.

2.4. Intracellular signaling analysis

After preincubation in serum-free M199 for 30 min, cardiomyocytes were stimulated with glucose transport agonists for 10 min (insulin) or 20 min (oligomycin). Stimulations were terminated by three rinses in ice-cold PBS before solubilizing cells in 200 μl lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH7.5), 1 mM EDTA, 0.5% sodium deoxycholate, 1% Igepal CA 630, Halt protease and phosphatase inhibitor Cocktail (Pierce, Thermo Scientific). Proteins (50 mg) from each sample were separated on 8% SDS–PAGE gels and transferred onto polyvinylidene difluoride membranes. Western blots were immunoprobed with antibodies for phosphorylated and total signaling intermediates. The following primary antibodies were used: SOCS-3, STAT5, phospho-STAT5 (Tyr^{694}), Akt, phospho-Akt (Ser^{473}), phospho-Akt (Thr^{308}), AMPKα, phospho-AMPKα (Thr^{172}) and phospho-(Ser/Thr)-Akt substrate from Cell Signaling Technologies, AS160 and phospho-PDH-E1α (Ser^{300}) from Millipore, GLUT1 and GLUT4 from Abcam and α-tubulin (clone B-5-1-2) and α-actinin (clone EA-53) from Sigma-Aldrich. Densitometric analysis of chemiluminescent signals captured on films was performed using the ImageJ software (National Institutes of Health, http://rsb.info.nih.gov/ij).

2.5. Statistics

Data are presented as mean ± SEM and were compared by two-way ANOVA (Prism 5, GraphPad Software) followed by Bonferroni's post hoc test. Dose–response curves for insulin were fitted with sigmoid curves by the least squares method. Differences were considered significant when $p<0.05$.

3. Results

3.1. Dose-dependent effect of CT-1 on insulin- and metabolic stress-stimulated glucose uptake

In order to determine the chronic effects of cardiotrophin-1 on glucose metabolism, isolated adult rat cardiomyocytes were incubated for 7 days with CT-1 at two concentrations (1 and 10 nM) bracketing the concentration in human plasma (5–6 nM [5]). Cardiomyocytes stimulated with increasing doses of insulin exhibited a dose-dependent response with a maximal glucose uptake at 10^{-7} M of insulin. In cardiomyocytes exposed to 1 nM CT-1 glucose transport was markedly reduced at all concentrations of insulin, indicating reduction in insulin responsiveness rather than in insulin sensitivity (Fig. 1A). CT-1 at 1 nM also reduced oligomycin-stimulated glucose transport (Fig. 1B). Intriguingly, we observed increased basal and insulin- and oligomycin-stimulated glucose uptake in cardiomyocytes treated with 10 nM CT-1, compared to control. Again insulin responsiveness but not insulin sensitivity was increased by treatment with 10 nM CT-1.

3.2. Cell signaling in cardiomyocytes exposed to 1 nM CT-1

CT-1 activates STAT1, STAT3 and STAT5 in adipocytes [9]. Investigations in STAT signaling in cardiomyocytes exposed to 1 nM CT-1 revealed a marked activation of STAT5, as assessed from phosphorylation on Tyr^{694} (Fig. 2A). In control cardiomyocytes, basal STAT5 phosphorylation was low, but increased in response to insulin, as previously reported [16,17], and to oligomycin, a new finding. In cardiomyocytes chronically exposed to 1 nM CT-1 STAT5 phosphorylation was markedly enhanced in all conditions.

STAT5 activation is known to enhance the expression of SOCS-3 [17–19], a negative regulator of insulin signaling [18,20,21]. We found that 1 nM CT-1 increased SOCS-3 expression, while the double treatment with 1 nM CT-1 and a cell-permeable STAT5 inhibitor [22] restored basal level of SOCS-3 (Figs. 2B and C).

In addition, we observed that 1 nM CT-1 markedly reduced the expression of GLUT4. Expression of GLUT4 was however restored in cardiomyocytes exposed to both CT-1 and the STAT5 inhibitor (Figs. 2B and D). GLUT1 expression was not altered by treatment with 1 nM CT-1 (Fig. 6C).

To determine whether STAT5 activation actually impairs stimulation of glucose transport, we measured basal and stimulated glucose