Original article

MOLECULAR METABOLISM

Pannexin 1 is required for full activation of insulin-stimulated glucose uptake in adipocytes

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ABSTRACT

Objective: Defective glucose uptake in adipocytes leads to impaired metabolic homeostasis and insulin resistance, hallmarks of type 2 diabetes. Extracellular ATP-derived nucleotides and nucleosides are important regulators of adipocyte function, but the pathway for controlled ATP release from adipocytes is unknown. Here, we investigated whether Pannexin 1 (Panx1) channels control ATP release from adipocytes and contribute to metabolic homeostasis.

Methods: We assessed Panx1 functionality in cultured 3T3-L1 adipocytes and in adipocytes isolated from murine white adipose tissue by measuring ATP release in response to known activators of Panx1 channels. Glucose uptake in cultured 3T3-L1 adipocytes was measured in the presence of Panx1 pharmacologic inhibitors and in adipocytes isolated from white adipose tissue from wildtype (WT) or adipocyte-specific Panx1 knockout (AdipPanx1 KO) mice generated in our laboratory. We performed *in vivo* glucose uptake studies in chow fed WT and AdipPanx1 KO mice and assessed insulin resistance in WT and AdipPanx1 KO mice fed a high fat diet for 12 weeks. Panx1 channel function was assessed in response to insulin by performing electrophysiologic recordings in a heterologous expression system. Finally, we measured Panx1 mRNA in human visceral adipose tissue samples by qRT-PCR and compared expression levels with glucose levels and HOMA-IR measurements in patients.

Results: Our data show that adipocytes express functional Pannexin 1 (Panx1) channels that can be activated to release ATP. Pharmacologic inhibition or selective genetic deletion of Panx1 from adipocytes decreased insulin-induced glucose uptake *in vitro* and *in vivo* and exacerbated diet-induced insulin resistance in mice. Further, we identify insulin as a novel activator of Panx1 channels. In obese humans Panx1 expression in adipose tissue is increased and correlates with the degree of insulin resistance.

Conclusions: We show that Panx1 channel activity regulates insulin-stimulated glucose uptake in adipocytes and thus contributes to control of metabolic homeostasis.

Keywords Pannexin 1; Adipocyte; Extracellular ATP; Glucose uptake

1. INTRODUCTION

Adipose tissue is renowned for its function in lipid storage, but it is also a key endocrine organ and metabolic dysfunction of adipocytes exacerbates insulin resistance [1]. In healthy adipose tissue, insulin stimulates glucose uptake and lipogenesis while inhibiting lipolysis, but these effects are blunted during insulin resistance [1]. Furthermore, glucose uptake in adipocytes is a major contributor to whole body insulin sensitivity [2].

The purine nucleoside adenosine accumulates extracellularly in isolated adipocyte suspensions [3–5] and is thought to be derived from degradation of extracellular ATP. Adenosine can impact adipocyte metabolic function by inhibiting lipolysis [6], increasing glucose uptake [7,8], enhancing insulin action to stimulate glucose oxidation [9], and regulating adipose tissue blood flow [10]. In addition to adenosine, extracellular nucleotides such as ATP or UTP have autocrine effects on adipocytes. They signal through the purinergic P2 receptor family, which includes ATP-gated cation channels $P2X_{1\text{-}7}$ and G-protein-coupled $P2Y_{1,2,4,6,11\text{-}14}$ receptors [11]. In brown adipose tissue, extracellular ATP caused mobilization of intracellular calcium stores, consistent with nucleotide signaling through purinergic P2 receptors [12]. Extracellular ATP also led to increased cell membrane capacitance in adipocytes [13,14], and it was suggested that ATP activates exocytosis [15]. In white adipocytes, micromolar concentrations of ATP inhibit glucose oxidation [16], but increase glycogen synthesis [17] and lipogenesis [18]. However, the effect of exogenous ATP on basal and insulin-induced glucose uptake in adipocytes is still a matter of controversy: ATP was shown in some studies to be inhibitory at millimolar

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concentrations [19,20], while in other work, no effect of ATP was observed [18].

Purinergic signaling in endocrine organs has been a major research focus [reviewed by Burnstock [21]], and novel therapies based on purinergic receptors as potential drug targets for type II diabetes have been suggested [22–24]. However, there are many purinergic receptors, and modulating ATP release could represent an alternative therapeutic strategy. In this respect, it remains an open question as to how nucleosides and nucleotides are released from adipocytes in a controlled manner.

Pannexin 1 (Panx1) channels control the release of ATP and other nucleotides from many cell types [25]. For example, activation of Panx1 channels initiates paracrine signaling that controls blood vessel constriction [26,27] and clearance of dying cells [28]. Pannexins (Panx1, 2, and 3) are hexameric membrane channels that are structurally similar to connexins but do not form gap-junctions [29]. Panx1 and 3 are present in many tissues while Panx2 is mainly expressed in the brain [29]. Activation of Panx1 can proceed by various mechanisms including α1-adrenergic stimulation [30], mechano-stretch [31], and caspase-mediated cleavage of the C-terminal portion of Panx1, an irreversible process [32]. Posttranslational modification including phosphorylation and S-nitrosylation was suggested to regulate Panx1 channel function [25] and known pharmacological inhibitors of Panx1 include carbenoxolone, probenecid, mefloquine, the food dye FD&C Blue No. 1, and trovafloxacin [33–36]. Here we describe an unexpected role for Panx1 channels, controlling ATP release from adipocytes, which is required for full activation of insulin-induced glucose uptake. Since dysregulation of glucose uptake in adipocytes was shown to impact whole body insulin sensitivity [37], Panx1 may play a key role in the pathophysiology of insulin resistance.

2. METHODS

2.1. Cell-based assays

For YO-PRO® dye uptake studies, 3T3-L1 cells were confluently plated in 6 well plates, differentiated into adipocytes as described, and treated with phenylephrine for 30 min followed by staining for 10 min with YO-PRO® (1 μM) and Hoechst (1 μg/mL). ATP concentrations in supernatants were measured using Promega Cell Titer Glo reagent. Luminescence was read on a Tecan Infinite M200 plate reader. For 3T3-L1 adipocyte glucose uptake, 3T3-L1 fibroblasts were plated to confluency in 12 well plates and differentiated into adipocytes as described. Cells were pretreated with carbenoxolone or probenicid as indicated for 20 min at which point insulin was added for 15 min. Glucose uptake was measured in isolated adipocytes as described [38].

2.2. Mice

$Panx1^{fl/fl}Adipoq^{Cre}$ mice were generated through appropriate breeding between the $Adipoq^{cre}$ mice [39] with $Panx1^{fl/fl}$ mice [36]. Littermate controls were used for all experiments. All animal studies were approved by the Animal Care and Use Committee at the University of Virginia. Mice were fed a high fat diet containing 60% cal from fat and 0.2% cholesterol (Bioserv) or normal chow (Teklad). Fat and lean masses were measured by EchoMRI™-100H Body Composition Analyzer. Glucose and insulin tolerance tests were performed in accordance with recommendations published by Ayala et al. [40]. For glucose tolerance test, mice were fasted for 6 h and then injected with 1 g/kg glucose i.p. and blood glucose levels were measured from tail blood by glucometer (OneTouch Ultra) over 2 h. For insulin tolerance test, mice were fasted 6 h and then injected with 0.75 U/kg insulin i.p. and blood glucose levels were measured from tail blood by glucometer (OneTouch Ultra) over 1 h. *In vivo* glucose uptake studies were performed as described [41]. In brief, mice were fasted 6 h followed by intraperitoneal injection of 2 g/kg glucose containing 10 μCi [^{3}H] 2-deoxy-D-glucose. Gastrocnemius muscle and perigonadal adipose tissue were collected 2 h post injection and snap frozen. 2-deoxyglucose uptake in tissues was determined by passing tissue homogenates over poly-prep chromatography columns with AG1-X8 resin (Bio-rad) and then calculating the difference in radioactive counts between total homogenate and column eluent, normalizing to specific activity of glucose as determined by serum samples processed with perchloric acid.

2.3. Electrophysiology

Patch clamping of 3T3-L1 adipocytes with active caspase 3 was performed as described previously [32]. Whole-cell recordings were made at room temperature using Axopatch 200B amplifier (Molecular Devices) with a bath solution composed of 140 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose (pH 7.3). Borosilicate glass patch pipettes (3–5 MΩ) were filled with an internal solution containing 30 mM tetraethylammonium chloride, 100 mM $CsMeSO_4$, 4 mM NaCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM HEPES, 10 mM EGTA, 3 mM ATP-Mg, and 0.3 mM GTP-Tris (pH 7.3). Ramp voltage commands were applied by using pCLAMP software and Digidata1322A digitizer (Molecular Devices). HEK293T cells were transiently transfected using Lipofectamine2000 (Invitrogen), and underwent serum depletion for 2–4 h before patch recording in order to reduce basal insulin receptor signaling. Basal Panx1 current was recorded, and then insulin (180 nM) was applied to the bath solution, followed by CBX (50 μM). Note that no CBX-sensitive current was observed in HEK293T cells without heterologously expressing Panx1 [32]. Constructs used in HEK293T heterologous system include mouse Panx1 wildtype construct [42,43], human Panx1(TEV) construct [32], and an EGFP-tagged human insulin receptor construct (Addgene) [44].

2.4. Human adipose tissue samples

Omental adipose tissue samples were obtained from patients undergoing bariatric surgery. All protocols and procedures were approved by the Institutional Review Board at the University of Virginia (IRB HSR #14180). HOMA-IR was calculated using the formula: HOMA-IR = fasting insulin × fasting glucose/405 [45].

2.5. Statistical analysis

Statistical analyses were performed with Graph Pad Prism (GraphPad, San Diego, CA). Student's t-test or ANOVA with post hoc comparison tests were used as appropriate. F test was performed in Prism to determine if variances were similar among groups.

3. RESULTS

3.1. Pannexin 1 channels are expressed and functional in adipocytes

The functional role of Pannexin 1 (Panx1) in adipose tissue has not been reported. To examine whether adipocytes express Panx1, we used immunohistochemistry. Panx1 protein expression was clearly observed on membranes of adipocytes (arrows) in adipose tissue from wild-type C57Bl6 mice, while the staining was absent in adipose tissue from *Panx1 knockout (KO)* mice (Figure S1A). To explore the functionality of Panx1 channels in adipocytes we performed experiments with cultured 3T3-L1 adipocytes and primary adipocytes isolated from wild-type or *Panx1 KO* mice, using known activators of Panx1 channel

