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Modulation of hepatic copper-ATPase activity by insulin and glucagon involves protein kinase A (PKA) signaling pathway

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ABSTRACT

Different studies have revealed copper imbalance in individuals suffering from diabetes and obesity, suggesting that regulation of glucose and/or fat metabolism could modulate cellular copper homeostasis. To test this hypothesis we investigated whether the key hormones of energy metabolism, insulin and glucagon, regulate the functional properties of the major hepatic copper-transporter, ATP7B (*i.e.*, copper-dependent ATPase activity). We demonstrated that insulin reverses the effect of copper and stimulates retrograde trafficking of ATP7B from the canalicular membranes, consistent with the enhanced ability of ATP7B to sequester copper away from the cytosol. Physiological concentrations of insulin increase endogenous ATP7B activity in cultured hepatic cells and in tissues by 40%, whereas glucagon inhibits this activity by 70%. These effects were cancelled out when insulin and glucagon were combined. We also demonstrated that the opposite effects of the hormones on ATP7B activity involve receptor-mediated signaling pathways and membrane-bound kinases (PKA and PKB/Akt), which are reciprocally regulated by insulin and glucagon. Inhibiting insulin signaling at the level of its Tyr-kinase receptor, PI3K or PKB/Akt restored the basal activity of ATP7B. Insulin reduced endogenous PKA activity, whereas glucagon promoted PKA stimulation by approximately 100%. These findings demonstrate that the physiological modulation of ATP7B activity is linked to energy metabolism via insulin and glucagon, and could help to understand the mechanisms involved in the disruption of copper homeostasis in diabetic and obese patients.

1. Introduction

Copper is an essential metal, a cofactor for several enzymes that are important in various metabolic processes. Although it is essential, copper is highly toxic because it can generate reactive oxygen species, so copper homeostasis is carefully regulated. In mammals, two Cu(I)-ATPases are important. ATP7A, which is present in almost all tissues, is responsible for copper absorption by enterocytes and the delivery of the metal to cuproenzymes. ATP7B, which is present in different organs, particularly in the liver, is responsible for excreting copper via the bile and for incorporating it into ceruloplasmin [1]. Under basal copper concentrations, ATP7A and ATP7B reside in the distal region of the Golgi complex (*trans*-Golgi network or TGN). When the intracellular copper concentration increases, ATP7A and ATP7B are transported from the *trans*-Golgi region to the plasma membrane. The functional significance of this translocation depends on the organ. In polarized kidney cells, ATP7A is located in the basolateral membrane and facilitates copper reabsorption [2], whereas in hepatocytes, ATP7B moves to the apical canalicular membrane to transport copper into the bile [3]. It is worth mentioning that trafficking of copper-dependent ATPases is widely distributed in biology, not only related to copper homeostasis but also involved in tissue remodeling, as in vascular smooth muscle cells under physiological and pathological conditions [4].

The activity and translocation of Cu(I)-ATPases are both tightly regulated by several post-translational processes, including kinase-mediated phosphorylation. There is a link between copper homeostasis

Abbreviations: TETA, triethylenetetramine hydrochloride salt; BCS, bathocuproinedisulphonic acid; PDE3B, 3B isoform of cyclic nucleotide phosphodiesterase.

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and protein kinase-mediated signaling events [1,5–10]. In mammals, hormones and/or autacoids can modulate copper transport by activating or inhibiting certain protein kinase-dependent pathways [11–13]. Of particular interest is the link between diabetes and abnormal copper balance in rats and humans, which could be due to an altered insulin/glucagon ratio, and consequently to kinase-mediated signaling through these two hormones. However the link is poorly studied regarding its mechanism. In diabetic humans, basal urinary copper excretion is higher than in control subjects, and TETA dihydrochloride (trientine; a copper chelator) stimulates excretion in both groups [14]. TETA is also important for protecting against pathogenic mechanisms in diabetic nephropathy [15]; it also alleviates heart failure and left ventricular injury in diabetic rats [16]. These observations support a role for the dysregulation of copper homeostasis in the genesis of these serious disorders arising from metabolic disturbances.

Therefore, it is plausible that insulin and glucagon, two important hormones that control energy metabolism, also participate in the modulation of copper homeostasis. Nevertheless, few if any published studies have reported the effect of glucagon on active copper transport. Although some studies have suggested the involvement of insulin in copper balance, the cellular and molecular mechanisms of this relationship are not well established. Treating polarized placental JEG-3 cells with insulin for 24 h stimulates the trafficking of ATP7A to the basolateral membrane but does not alter the location of ATP7B in the Golgi complex [12]. In a model of streptozotocin-induced diabetes, ATP7A is expressed at a lower level than in control rats, and its expression returns to control levels following treatment with insulin [17]. However, these approaches do not address the modulation of Cu(I)-ATPase activity. Therefore, we investigated whether: (i) insulin and glucagon influence *ATP7B* gene transcription and ATP7B protein location; (ii) these hormones modulate ATP7B activity, and if so, (iii) which signaling pathways and protein kinase(s) are involved.

2. Materials and methods

2.1. Materials

Buffers, protease inhibitors, ATP, insulin, glucagon, GTPγS, $PKAi_{5–24}$, Akt1/2 kinase inhibitor, cilostamide, anti-Golgi 58K antibody, and Coon's modified F12 medium were supplied by Sigma Aldrich. Phospho-Akt (Ser^{473}) and Akt antibodies were supplied by Cell Signaling. HPRT-conjugated secondary antibody was supplied by GE Healthcare. Protease inhibitor cocktail tablets were supplied by Roche. Absolutely RNA Miniprep Kit and Affinity Script qPCR cDNA Synthesis Kit were from by Agilent Technologies. $^{32}P_i$ was obtained from the Instituto de Pesquisas Energéticas e Nucleares (IPEN). [γ-^{32}P]ATP was prepared as described by Maia et al. [18].

2.2. Cell culture

WIF-B9 cells [19] were grown in Coon's modified F12 medium supplemented with HAT (10^{-5} M hypoxanthine, 4×10^{-8} M aminopterin, 1.6×10^{-4} M thymidine), 5% FBS, 2 mM glutamine and an antibiotic and antifungal solution containing 100 μg/ml streptomycin, 100 U/ml penicillin and 0.25 μg/ml amphotericin B. HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% *v*/*v* fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 U/ml penicillin. Cells were cultured at 37 °C in a humidified air atmosphere with 5% (HepG2) or 7% (WIF-B9) CO_2. The culture medium was renewed every 2–3 days.

2.3. ATP7B localization by immunofluorescence

WIF-B9 cells were cultured and examined by immunofluorescence as described in [20]. Cells were grown on glass coverslips and rinsed three times with 1 ml PBS. After permeabilization with methanol for 4 min at −20 °C, they were rehydrated with PBS and incubated with a rabbit primary antibody, anti-ATP7B (1:200), or p58 anti-Golgi 58 K (1:300), for 45 min at 37 °C. The coverslips were rinsed three times with PBS, and cells that had been treated with anti-ATP7B were incubated for 20 min at 37 °C with Alexa 488-conjugated goat anti-rabbit secondary antibody (1:500; Molecular Probes). Cells that had been treated with p58 anti-Golgi were incubated with Alexa 568-conjugated anti-mouse secondary antibody for 20 min at 37 °C (1:1000). All the cells were washed three times with PBS and the coverslips were placed on glass slides with mounting medium (Sigma) and sealed using nail polish. The rabbit primary antibody anti-ATP7B (a gift from Dr. Ignacio Sandoval, CIBEREHD, Madrid, Spain) was directed against the N-terminal 655 residues of human ATP7B [21]. Cells were examined using a Zeiss Axioskop fluorescence microscope.

ATP7B trafficking was analyzed as previously described [20]. Different microscopic fields from at least two different experiments were observed using phase contrast (to visualize the canaliculi) and immunofluorescence microscopy. For each concentration of insulin, 3–5 microscopic fields from each experiment were examined to count the number of canaliculi per field. The percentage fluorescence labeling for ATP7B in each field was calculated as follows: (i) only in the canaliculi; (ii) between the canaliculi and the Golgi; (iii) only in the Golgi. The percentage of ATP7B-encircled canaliculi was calculated using the ratio $[(i) / (i + ii + iii)] \times 100$.

Localization of ATP7B with respect to p58 (the Golgi marker) was quantified as follows. The ratio between ATP7B fluorescence area and p58 fluorescence area was obtained in the same individual cells using ImageJ software: Twenty-five cells were examined from a different set of experiments using 3 different cultures under control, insulin and glucagon conditions. The ATP7B and p58 fluorescence integrated areas were corrected by simultaneous measurement of the background in 3 random areas with no fluorescence in the same field. The precise localization of ATP7B was confirmed by merge images before each measure.

2.4. Cell membrane preparation

WIF-B9 or HepG2 cells were incubated for different times in serum-free culture medium supplemented with insulin, glucagon or H89, as indicated in the figures. After incubation, the medium was discarded and the cells were washed twice with HB (pH 7.3), then scraped and centrifuged for 10 min at 2400 ×*g* at 4 °C. The pellet containing distinct membranes (nucleus, endoplasmic reticulum, *trans*-Golgi complex and plasma membrane) was resuspended in HB and homogenized in a Potter-Teflon system. After centrifugation for 15 min at 16,000 ×*g*, 4 °C, the pellet was re-extracted with 100 mM MOPS-KOH (pH 7.3), homogenized, and centrifuged again under the same conditions. The supernatant was decanted and the pellet was resuspended in the same buffer. ATP7B activity was measured within the same day, as described below.

2.5. Real-time PCR

Total cellular RNA was extracted from HepG2 cells using Absolutely RNA Miniprep Kit (Agilent Technologies). One microgram of total RNA was reverse transcribed using the Affinity Script qPCR cDNA Synthesis Kit (Stratagene Agilent). Amplification reactions were performed using SYBR Green PCR Master Mix and 200 nM of forward and reverse primers for ATP7B (5′-TGGCAAAGTCCCCACAATCAAC-3′; 5′-TCAAGGCAACCAAC ACGGAGAG-3′). Samples were analyzed in triplicate in a 10-μl reaction volume using the Cfx 96 Bio-Rad. The internal controls were 36B4 (RPLP0 Ribosomes) and HPRT (hypoxanthine phosphoribosyltransferase). The abundance of each transcript was measured by determining the CT (threshold cycle) value after each reaction according to reference [22].