Stimulation of Electrogenic Glucose Transport by Glycogen Synthase Kinase 3

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Key Words

Dual electrode voltage clamp • SGLT1 • Intestine • Ussing chamber

Abstract

Glycogen synthase kinase 3 GSK3 β participates in a wide variety of functions including regulation of glucose metabolism. It is ubiquitously expressed including epithelial tissues. However, whether GSK3 β participates in the regulation of epithelial transport is not known. The present study thus explored whether GSK3 β influences the Na⁺-coupled transport of glucose. To this end, SGLT1 was expressed in *Xenopus* oocytes with or without GSK3 β and glucose-induced current (I_g) determined by dual electrode voltage clamp. In *Xenopus* oocytes expressing SGLT1 but not in water-injected oocytes glucose induced an inwardly directed I_g , which was significantly enhanced by coexpression of GSK3 β . According to chemiluminescence and confocal microscopy, GSK3 β increased the SGLT1 protein abundance in the oocyte cell membrane. To explore whether GSK3 β sensitivity of SGLT1 participates in the regulation of electrogenic intestinal glucose transport, Ussing chamber experiments were performed in intestinal

segments from gene-targeted knockin mice with mutated and thus PKB/SGK-resistant GSK3 α,β (*gsk3^{KI}*), in which the serine of the PKB/SGK phosphorylation site was replaced by alanine, and from wild type mice (*gsk3^{WT}*). The glucose-induced current was significantly larger in *gsk3^{KI}* than in *gsk3^{WT}* mice. The present observations reveal a novel function of GSK3, i.e. the stimulation of Na⁺-coupled glucose transport.

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Introduction

Glycogen synthase kinase GSK3, an ubiquitously expressed signaling molecule, was originally discovered as a kinase participating in the regulation of glycogen synthesis but later shown to be involved in the regulation of a wide variety of cellular functions [1, 2]. The two isoforms GSK3 α and GSK3 β are encoded by distinct genes but largely overlap in regulators and phosphorylation targets [3]. GSK3 may play a role in a variety of pathophysiological conditions, including diabetes mellitus, inflammation, tumor growth, mood disorders, memory consolidation, neurodegeneration and ischemia [3-5]. The kinase is phosphorylated and thus inhibited by the Wnt

pathway [2], by protein kinase Akt/PKB [6-8] and the serum and glucocorticoid-inducible kinase SGK [9, 10].

In order to elucidate the physiological impact of PKB/SGK-dependent regulation of GSK3, gene-targeted mice were generated, in which the serine within the PKB/SGK phosphorylation site was replaced by alanine (GSK3 $\alpha^{21A/21A}$, GSK3 $\beta^{9A/9A}$) thus yielding resistance of GSK3 to inactivation by PKB/SGK [11]. As expected, the knockin mice carrying these mutations (*gsk3^{KI}*) are resistant to the effect of insulin on muscle glycogen synthase [11]. Properties of *gsk3^{KI}* mice include increased body temperature, blood pressure, food and water intake, fecal excretion, glomerular filtration rate, urinary flow rate, urine osmolality, urinary Na⁺, K⁺ & urea excretion as well as proteinuria [12, 13].

SGK sensitive functions include stimulation of SGLT1 [14], the carrier accounting for the electrogenic intestinal glucose transport [15]. Accordingly, electrogenic glucose transport is decreased in mice lacking functional SGK1 [16] and SGK3 [17]. At least in theory, inhibition of GSK3 could have participated in the stimulation of intestinal glucose transport by the SGK isoforms.

The present study thus explored the putative role of GSK3 in the regulation of intestinal glucose transport. Experiments were performed in animals expressing PKB/SGK insensitive GSK3 α and GSK3 β . Surprisingly, glucose transport in these mice was enhanced, suggesting that GSK3 stimulates glucose transport. Thus, additional experiments were performed in *Xenopus* oocytes utilizing the isoform GSK3 β .

Materials and Methods

In vitro experiments

For the generation of cRNA, constructs were used encoding wild type human SGLT1 [14] and wild type human GSK3 β [2]. The cRNA was generated as described previously [18, 19]. For analyzing SGLT1 activity by dual electrode voltage clamp, *Xenopus laevis* oocytes were prepared as previously described [20, 21]. 7.5 ng cRNA encoding constitutively active S422D SGK1 was injected on the first day, then 7.5 ng of wild type GSK3 β and 5 ng SGLT1 cRNA were injected on the second day after preparation of the *Xenopus* oocytes. All experiments were performed at room temperature 3-4 days after the second injection. Two-electrode voltage-clamp recordings were performed at a holding potential of -70 mV. The data were filtered at 10 Hz, and recorded with a GeneClamp 500 amplifier, a DigiData 1300 A/D-D/A converter and the pClamp 9.0 software package for data acquisition and analysis (Axon Instruments, USA). The control solution (superfusate / ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM

HEPES, pH 7.4. Glucose was added to the solutions at the indicated concentrations. The final solutions were titrated to pH 7.4 using NaOH (all substances were from Sigma, Schnelldorf, Germany, or from Roth, Karlsruhe, Germany).

The flow rate of the superfusion was 20 ml/min and, in theory, a complete exchange of the bath solution was reached within about 10 s. However, due to unstirred layers the full exchange of fluid around the oocyte may have been slower.

Immunohistochemistry was employed to determine carrier protein abundance in the cell membrane [22]. After 4% paraformaldehyde fixation for at least 12 h, oocytes were cryoprotected in 30% sucrose, frozen in Tissue Tek[®] O.C.T.™ Compound (Sakura Finetek) and placed on a cryostat. Sections were collected at a thickness of 8 μ m on coated slides and stored at -20°C. For immunostainings, sections were dehydrated at RT, fixated in acetone/methanol (1:1) for 15 minutes at RT, washed in PBS and pre-incubated for 1 h in 5% bovine serum albumin in PBS. The anti-SGLT1 antibody (rabbit anti-SGLT1, Millipore, United States) was diluted 1:1000 and applied in a moist chamber overnight at 4°C. Binding of anti-SGLT1 antibody was visualized with an anti-rabbit conjugated Cy3 antibody (1:1000, donkey anti-rabbit, Millipore, United States). Slides were analyzed on a confocal laser-scanning microscope (Zeiss LSM 510 Exciter, Germany) using appropriated filter sets.

For detection of cell surface expression by chemiluminescence, defolliculated oocytes were incubated with 1 μ g/ml primary mouse monoclonal anti-glucose transporter SGLT1 antibody (rabbit anti-SGLT1, Millipore, United States) and 2 μ g/ml secondary, peroxidase-conjugated sheep anti-rabbit IgG antibody (GE Healthcare, Amersham, United Kingdom). Individual oocytes were placed in 20 μ l of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, USA), and chemiluminescence was quantified in a luminometer by integrating the signal over a period of 1 s. Integrity of the measured oocytes was assessed by visual control after the measurement to avoid unspecific light signals from the cytosol.

For quantitative real-time PCR total RNA was isolated from intestinal tissue by using the Qiashredder and RNeasy Mini Kit (Quiagen, Hilden, Germany). Random hexamers (Roche Diagnostics, Penzberg, Germany) and Super ScriptII reverse transcriptase (Invitrogen) were used for reverse transcription of total RNA. SGLT1 levels were determined using quantitative RT-PCR with the CFX96™ Real-Time System (Bio-Rad).

For *SGLT1* the primers 5' GTG GTA CCG TTG GAG GCT TC 3' (forward) and 5' CCA CTT CCA ATG TTA CTG GCAA 3' (reverse) and for *Tbp* 5' CAC TCC TGCC ACA CCA GCT T 3' (forward) and 5' TGG TCT TTA GGT CAA GTT TAC AGC C 3' (reverse) were used. PCR reactions were performed in a final volume of 20 μ l containing 2 μ l cDNA, 2 μ l of each primer (0.5 μ M), 2 μ l cDNA Master SybrGreen I mix (Roche Molecular Biochemicals, Mannheim, Germany) and 10 μ l sterile water. Reactions were run for 40 cycles consisting of 30 seconds denaturation at 95°C, primer annealing for 30 seconds at 55 °C, and extension for 30 seconds at 72 °C. Amplification of the housekeeping gene *TATA Box binding-protein* (*Tbp*) was performed to standardize the amount of sample RNA. Relative quantification of gene expression was performed using the $\Delta\Delta$ Cp method.

Ex vivo experiments

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities.

Mice were generated, in which the codon encoding Ser9 of GSK3 β gene was changed to encode nonphosphorylatable alanine (GSK3 $\beta^{9A/9A}$), and simultaneously the codon encoding Ser21 of GSK3 α was changed to encode the nonphosphorylatable GSK3 $\alpha^{21A/21A}$ thus yielding the GSK3 $\alpha/\beta^{21A/21A/9A/9A}$ double knockin mouse (*gsk3^{KT}*) as described previously [11]. The mice were compared to age and gender-matched wild type mice (*gsk3^{WT}*).

The mice (6 females, 7 males, age 6-12 months) were fed a control diet (1314, Altromin, Heidenau, Germany). The mice had free access to tap drinking water.

For analysis of electrogenic intestinal glucose transport, jejunal segments were mounted into a custom made mini-Ussing chamber with an opening of 0.00769 cm². Under control conditions, the serosal and luminal perfusate contained (in mM): 115 NaCl, 2 KCl, 1 MgCl₂, 1.25 CaCl₂, 0.4 KH₂PO₄, 1.6 K₂HPO₄, 5 Na pyruvate, 25 NaHCO₃ (pH 7.4, NaOH). Where indicated, glucose (20 mM) was added to the luminal perfusate at the expense of mannitol (20 mM) (all substances were from Sigma, Schnellendorf, Germany, or from Roth, Karlsruhe, Germany).

In all Ussing chamber experiments the transepithelial potential difference (Vt) was determined continuously and the transepithelial resistance (Rt) was estimated from the voltage deflections (ΔV_t) elicited by imposing test currents (I_t). The resulting Rt was calculated according to Ohm's law.

Statistics

Data are provided as means $\pm$ SEM, *n* represents the number of independent experiments. All data were tested for significance using Student t-test or ANOVA, as applicable and only results with *p* < 0.05 were considered statistically significant.

Results

In an attempt to elucidate the effect of the glycogen synthase kinase 3 β on the sodium-coupled glucose transporter SGLT1, cRNA encoding SGLT1 was injected into *Xenopus laevis* oocytes with or without additional injection of cRNA encoding GSK3 β . Electrogenic glucose transport was estimated from the glucose-induced current. In oocytes injected with water the addition of glucose was not followed by a significant current indicating that *Xenopus laevis* oocytes do not express a sizeable electrogenic glucose transporter (Fig. 1A). The expression of GSK3 β alone was similarly not followed by the appearance of a glucose-induced current. In contrast, addition of glucose induced an inward current in *Xenopus laevis* oocytes expressing SGLT1. The current was significantly increased by the additional expression of

Fig. 1. Glucose-induced current in SGLT1-expressing *Xenopus* oocytes. A. Original tracings (upper panel) and arithmetic means $\pm$ SEM (*n* = 6-34, lower panel) of glucose- (10 mM) induced currents in *Xenopus* oocytes injected with water (outer left bar) or cRNA encoding GSK3 β (inner left bar), cRNA encoding SGLT1 (inner right bar) and SGLT1 + GSK3 β cRNA (outer right bar). * indicates significant difference from absence of cRNA encoding GSK3 β (*p* < 0.05). B. Arithmetic means $\pm$ SEM (*n* = 11-27) of glucose- (10 mM) induced currents in *Xenopus* oocytes injected with water (1st bar) or with cRNA encoding SGLT1 (2nd - 5th bar) without (2nd, 4th bar) or with (3rd, 5th bar) cRNA encoding constitutively active S422D SGK1 in the absence (white bars) or presence (black bars) of cRNA encoding GSK3 β . **, *** indicate significant difference from currents determined in oocytes only injected with SGLT1 cRNA (*p* < 0.01, *p* < 0.001).

Fig. 2. GSK3 β increases the maximal current but not the affinity of SGLT1. Arithmetic means $\pm$ SEM (*n* = 14-16) of currents induced by glucose at the indicated concentrations (0.05 to 100 mM) in *Xenopus* oocytes injected with cRNA encoding SGLT1 without and with additional injection of cRNA encoding GSK3 β . *, ** indicate significant difference from the absence of cRNA encoding GSK3 β (*p* < 0.05, *p* < 0.01).

Fig. 3. Coexpression of GSK3 β enhances the SGLT1 protein abundance within the plasma membrane of oocytes. A. Confocal images of SGLT1 protein abundance in the plasma membrane of *Xenopus* oocytes injected with water (left), injected with SGLT1 alone (middle) and injected with SGLT1 and GSK3 β (right). B. Arithmetic means $\pm$ SEM (n = 3) of surface SGLT1 expression assessed by chemiluminescence in oocytes injected with water (left), expressing SGLT1 alone (middle) or together with GSK3 β (right). Cell surface expression was normalized to the mean relative light units value obtained in oocytes expressing SGLT1 alone. * indicates statistically significant difference from *Xenopus* oocytes expressing SGLT1 alone.

GSK3 β (Fig. 1A). Further experiments aimed to test whether the effect of GSK3 β is modified by the additional expression of constitutively active ^{S422D}SGK1. As shown in Fig. 1B, expression of ^{S422D}SGK1 in SGLT1-expressing oocytes resulted in the expected significant increase in the glucose-induced current compared to only SGLT1-expressing oocytes. Coexpression of ^{S422D}SGK1 along with GSK3 β tended to increase the SGLT1-mediated current compared to SGLT1-expressing oocytes injected with cRNA encoding GSK3 β alone, an effect, however not reaching statistical significance.

The increase in glucose-induced current could have been due to an increase in the maximal transport rate or due to an increase in the affinity of the carrier. The currents induced by the addition of different concentrations of glucose allowed the calculation of an apparent maximal transport rate ($V_{\max}$) and the concentration needed for half maximal current (K_M). As illustrated in Fig. 2, the coexpression of GSK3 β increased $V_{\max}$ (from 70 ± 1 nA to 108 ± 1 nA, n = 14-16) but did not significantly alter K_M (656 ± 27 μ M without and 647 ± 21 μ M with coexpression of GSK3 β , n = 14-16).

An increase in the maximal transport rate could have resulted from an increase in SGLT1 protein abundance in the cell membrane. To test that possibility, the SGLT1 protein abundance was determined by confocal microscopy and by chemiluminescence in oocytes injected with water, in oocytes expressing SGLT1 alone and in oocytes expressing SGLT1 together with GSK3 β . As shown in Fig. 3, the SGLT1 cell surface expression was significantly increased by the coexpression of GSK3 β in

Fig. 4. Glucose-induced transepithelial current (I_g) in intestine. Glucose-induced alterations of transepithelial voltages (ΔV_t) and glucose-induced currents (I_g) in jejunal segments from GSK3 knockin mice (*gsk3^{KI}*) and wild type mice (*gsk3^{WT}*). A: Original tracings from typical experiments illustrating the effect of 20 mM glucose on the transepithelial potential difference in *gsk3^{KI}* and *gsk3^{WT}* mice. B: Arithmetic means $\pm$ SEM (n = 11) of glucose-induced currents in jejunum from *gsk3^{WT}* (open bar) and *gsk3^{KI}* (closed bar) mice. * indicates statistically significant difference (p < 0.05) between *gsk3^{KI}* and *gsk3^{WT}* mice.

oocytes injected with cRNA encoding SGLT1.

To elucidate the *in vivo* significance of GSK3-dependent regulation of electrogenic glucose transport,

jejunal segments from PKB/SGK-resistant GSK3 knockin mice (*gsk3^{KI}*) and wild type mice (*gsk3^{WT}*) were mounted into mini Ussing chambers and electrogenic glucose transport determined utilizing electrophysiological analysis. In the absence of luminal substrates, the transepithelial potential difference (Vt) amounted to 1.86 ± 0.07 mV ($n = 11$) in *gsk3^{WT}* mice and to 1.77 ± 0.14 mV ($n = 11$) in *gsk3^{KI}* mice. The transepithelial resistance (Rt) approached $6.69 \pm 0.42 \Omega \cdot \text{cm}^2$ ($n = 11$) in *gsk3^{WT}* mice and $6.09 \pm 0.97 \Omega \cdot \text{cm}^2$ ($n = 11$) in *gsk3^{KI}* mice. Neither the transepithelial potential difference nor the transepithelial resistance were significantly different between *gsk3^{KI}* and *gsk3^{WT}* mice.

The partial isosmotic replacement of mannitol by glucose (20 mM) created a lumen-negative shift of the transepithelial potential difference (ΔV_p) without significantly altering the transepithelial resistance. The ΔV_t was significantly higher in *gsk3^{KI}* than in *gsk3^{WT}* mice. ΔV_t and Rt allowed the calculation of the glucose-induced current (Ig), which was again significantly higher in *gsk3^{KI}* than in *gsk3^{WT}* mice (Fig. 4).

In theory, enhanced SGLT1-mediated intestinal glucose uptake of *gsk3^{KI}* mice could be due to transcriptional upregulation of SGLT1. To test for this possibility, quantitative RT-PCR was performed in intestinal tissue from *gsk3^{WT}* and *gsk3^{KI}* mice. The transcript levels were 157.9 ± 16.3 ($n = 6$) arbitrary units in *gsk3^{WT}* and 136.1 ± 26.2 ($n = 6$) arbitrary units in *gsk3^{KI}* mice, values, not significantly different.

Discussion

The present study reveals a completely novel function of GSK3, i.e. the stimulation of the Na⁺-coupled glucose transporter SGLT1. GSK3 is effective through upregulation of SGLT1 protein abundance in the cell membrane. The effect on transport is seemingly less pronounced. In theory, GSK3 may exert dual effects, i.e. upregulation of carrier protein abundance and negative influence on the activity of the carrier. However, the scatter of data and the uncertainties during comparisons of data obtained with different methodologies preclude safe conclusions. No attempts have been made to discriminate between GSK3 α and GSK3 β . The two kinases are similar in regulation and targets [11].

The stimulation of SGLT1 by GSK3 is somewhat counterintuitive, as the GSK3 is phosphorylated and thus inhibited by protein kinase B Akt/PKB [6-8] and the

serum and glucocorticoid-inducible kinase SGK [9, 10], both kinases well known to stimulate a variety of transport systems [23-26]. According to the present observations, the inhibition of GSK3 by PKB/Akt and SGK isoforms would be expected to decrease the SGLT1 transport activity, the opposite, of what is observed [14]. Obviously, Akt/PKB stimulate glucose transport via other mechanisms than inhibition of GSK3 β . SGK1 and Akt/PKB are at least partially effective on SGLT1 by phosphorylation of the ubiquitin ligase Nedd4-2 [14], which otherwise ubiquitinates target proteins including SGLT1 thus preparing them for degradation.

The experiments in oocytes are supported by the observations in mice expressing protein kinase B (PKB)/serum and glucocorticoid-inducible kinase (SGK)-resistant glycogen synthase GSK3. The GSK3 isoforms were made resistant to PKB/SGK isoforms by introducing a mutation of GSK3 β in which the serine of the PKB phosphorylation site was replaced by an alanine (GSK3 β^{9A}) and at the same time a mutation in GSK3 α in which the serine of the PKB phosphorylation site was replaced by an alanine (GSK3 $\beta^{12A/12A}$). In those mice GSK3 activity is not under the inhibitory influence of Akt/PKB and SGK and is thus enhanced [11]. The PKB/SGK resistance of GSK3 leads to enhanced activity of SGLT1. Accordingly, the glucose-induced current in intestinal segments is larger in mice carrying the PKB/SGK resistant GSK mutants (*gsk3^{KI}*) than in wild type mice (*gsk3^{WT}*).

Inhibition of GSK3 further mediates the effect of insulin on glycogen synthase [11, 27]. Accordingly, the stimulating effect of insulin on muscle glycogen synthase is abrogated in *gsk3^{KI}* mice [11].

According to the present observations, the inhibitory effect of SGK and Akt/PKB isoforms on GSK3 is expected to blunt the stimulating effect of those kinases on SGLT1 protein abundance and activity. Under the experimental conditions employed previously, the effect of SGK and Akt/PKB isoforms on Nedd4-2 apparently override the effect on GSK3 and the additional regulation of SGLT1 by GSK3 appears to be futile. It must be pointed out that GSK3 is regulated not only by SGK and Akt/PKB isoforms but by additional signalling pathways including the Wnt [2], a regulation distinct from Akt/PKB dependent phosphorylation of the kinase [11]. Moreover, GSK3 has been shown to upregulate Akt/PKB expression [28]. The inhibition of GSK3 by Akt/PKB may thus be part of a negative feedback, which is disrupted in mice carrying Akt/PKB resistant GSK3. The influence of GSK on SGLT1 is expected to tune the gain of SGK and Akt/PKB dependent regulation of the carrier. In any case,

the present study sheds additional light on the complexity of insulin dependent transport regulation, which is sensitive to regulators of the GSK3 isoforms.

In conclusion, insensitivity of GSK3 to the inhibitory action of PKB and SGK leads to enhanced SGLT1 activity and thus to enhanced electrogenic intestinal glucose transport.

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