Original Paper

Down-Regulation of the Myoinositol Transporter SMIT by JAK2

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Inositol transport • Osmolyte • Cell volume • Energy depletion

Abstract

Background/Aims: Janus-activated kinase-2 JAK2 is activated by energy depletion and hyperosmotic shock and modifies the activity of several Na⁺ coupled transporters. The Na⁺ coupled osmolyte transporter SMIT (myoinositol transporter) is upregulated by osmotic shock and downregulated by energy depletion. The present study thus explored whether JAK2 contributes to the regulation of SMIT activity. **Methods:** To this end, cRNA encoding SMIT was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding wild type JAK2, constitutively active ^{V617F}JAK2 or inactive ^{K882E}JAK2. Inositol-induced current (I_{SMIT}) was determined by dual electrode voltage clamp and taken as measure for electrogenic inositol transport. **Results:** No appreciable I_{SMIT} was observed in water injected oocytes. In SMIT expressing oocytes I_{SMIT} was significantly decreased by additional coexpression of JAK2 or ^{V617F}JAK2, but not by coexpression of ^{K882E}JAK2. According to kinetic analysis coexpression of JAK2 decreased maximal I_{SMIT} without significantly modifying the concentration required for halfmaximal I_{SMIT} (K_M). In oocytes expressing both, SMIT and JAK2, I_{SMIT} was gradually increased by JAK2 inhibitor AG490 (40 μ M). Disruption of carrier insertion with brefeldin A (5 μ M) was followed by a decline of I_{SMIT} to a similar extent in *Xenopus* oocytes expressing SMIT with JAK2 and in *Xenopus* oocytes expressing SMIT alone, suggesting that JAK2 did not affect carrier stability in the cell membrane. **Conclusion:** JAK2 contributes to the regulation of the inositol transporter SMIT.

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Introduction

The janus-activated kinase-2 JAK2 participates in the signaling of several hormones and cytokines [1-3] including leptin [4], growth hormone [5, 6], erythropoietin [3], thrombopoietin [3] and granulocyte colony-stimulating factor [3]. Excessive JAK2 activity fosters the development of malignancy and JAK2 inhibitors may be effective in the treatment of myeloproliferative disorders [7-12]. The gain of function mutation ^{V617F}JAK2 presumably contributes to the pathogenesis of myeloproliferative disease [13-16].

JAK2 is activated by oxidative stress & ischemia [17] and by hypertonicity [18, 19] and has most recently been shown to up-regulate the betaine/GABA transporter BGT1 (SLC6A12) [20], an osmolyte transporter participating in the regulation of cell volume [21-23]. Another Na⁺ coupled osmolyte transporter is the myoinositol transporter SMIT, which accumulates myoinositol in osmotically shrunken cells [21, 24]. SMIT is genomically up-regulated by osmotic cell shrinkage, neuronal excitation and radiation [21-23, 25-39], an effect involving the transcription factor NFAT5 [40]. Little is known about posttranslational regulation of SMIT activity.

The present study explored whether JAK2 regulates the activity of SMIT. To this end, SMIT was expressed in *Xenopus* oocytes with or without wild type JAK2, constitutively active ^{V617F}JAK2 or inactive ^{K882E}JAK2 and inositol transport estimated from inositol induced current.

Materials and Methods

Constructs

Constructs were used encoding wild type SMIT [41], wild-type human JAK2 (Imagenes, Berlin, Germany), inactive ^{K882E}JAK2 mutant [42] and overactive ^{V617F}JAK2 mutant [13] generated by site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit; Stratagene, Heidelberg, Germany) according to the manufacturer's instructions [43]. The constructs were used for generation of cRNA as described previously [44].

Voltage clamp in *Xenopus* oocytes

Xenopus oocytes were prepared as described [45] Where not indicated otherwise, 25 ng SMIT cRNA were injected on the first day and 10 ng of wild type JAK2 cRNA on the second day or at the same day after preparation of the oocytes [46]. The oocytes were maintained at 17°C in a solution containing (in mM): 96 NaCl, 4 KCl, 1.8 MgCl₂, 0.1 CaCl₂, 5 HEPES, pH 7.4, gentamycin (50 mg/l), tetracycline (50 mg/l), ciprofloxacin (1.6 mg/l), refobacin (100 mg/l), and theophiline (90 mg/l). Where indicated, the JAK2 inhibitor AG490 (40 μM), brefeldin A (5 μM) or actinomycin D (10 μM) were added to the respective solutions. The voltage clamp experiments were performed at room temperature 7-10 days after injection [47]. Two-electrode voltage-clamp recordings were performed at a holding potential of -60 mV. The data were filtered at 10 Hz and recorded with a Digidata A/D-D/A converter and Clampex 9.2 software for data acquisition and analysis (Axon Instruments) [48]. The control superfusate contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES, pH 7.4. Inositol was added to the solutions at a concentration of 1 mM, unless otherwise stated. The flow rate of the superfusion was approx. 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s.

Statistical analysis

Data are provided as means ± SEM, n represents the number of oocytes investigated. All experiments were repeated with at least 2-3 batches of oocytes; in all repetitions qualitatively similar data were obtained. Data were tested for significance using ANOVA or t-test, as appropriate. Results with p < 0.05 were considered statistically significant.

Fig. 1. Coexpression of wild type JAK2 or active V_{617F} JAK2 but not of inactive mutant K_{882E} JAK2 decreases electrogenic inositol transport in SMIT-expressing *Xenopus* oocytes. A: Representative original tracings showing inositol (1 mM) - induced current (I_{SMIT}) in *Xenopus* oocytes injected with water (a), or expressing wild type JAK2 alone (b) SMIT alone (b), SMIT with wild type JAK2 (c), SMIT with constitutively active V_{617F} JAK2 (d), or SMIT with the inactive mutant K_{882E} JAK2 (e). B. Arithmetic means $\pm$ SEM (n = 20 - 27) of inositol (1 mM) - induced current (I_{SMIT}) in *Xenopus* oocytes injected with water (H_2O , dotted bar), or expressing SMIT without (SMIT, white bar) or with wild type JAK2 (SMIT + JAK2, dark grey bar), with constitutively active V_{617F} JAK2 (SMIT + V_{617F} JAK2, black bar) or with the inactive mutant K_{882E} JAK2 (SMIT + K_{882E} JAK2, light grey bar). *** indicates statistically significant ($p < 0.001$) difference to expression of SMIT alone.

Fig. 2. JAK2 decreases maximal transport rate of inositol transport in SMIT-expressing *Xenopus* oocytes. Arithmetic means $\pm$ SEM (n = 9) of inositol -induced current (I_{SMIT}) as a function of inositol concentration in *Xenopus* oocytes expressing SMIT without (closed circles, solid line), or with (open circles, dashed line) additional coexpression of wild type JAK2.

Results

To explore, whether Janus kinase-2 (JAK2) modifies the function of the myoinositol transporter (SMIT), cRNA encoding SMIT was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding JAK2. Electrogenic transport of inositol was apparent from inositol (1 mM) induced inward current (I_{SMIT}). No appreciable current was observed following exposure of non-injected or water-injected *Xenopus* oocytes to 1 mM inositol. Thus, *Xenopus* oocytes do not express appreciable endogenous electrogenic inositol transport (Fig. 1A,B). A sizable I_{SMIT} was, however, observed in *Xenopus* oocytes injected with cRNA encoding SMIT. The additional injection of cRNA encoding wild type JAK2 was followed by a significant decrease of I_{SMIT} in SMIT expressing *Xenopus* oocytes (Fig. 1A,B). The effect of wild type JAK2 was mimicked by the gain of function mutant V_{617F} JAK2 but not by the inactive mutant K_{882E} JAK2 (Fig. 1A,B). Accordingly, I_{SMIT} was significantly lower in *Xenopus* oocytes expressing SMIT together with V_{617F} JAK2 than in *Xenopus* oocytes expressing SMIT alone. In contrast, I_{SMIT} was similar in *Xenopus* oocytes expressing SMIT together with K_{882E} JAK2 and in *Xenopus* oocytes expressing SMIT alone. The effect of V_{617F} JAK2 coexpression tended to be lower than that of wild type JAK2 coexpression.

Kinetic analysis was performed to determine, whether JAK2 influenced the maximal transport rate or the affinity of the carrier. As illustrated in Fig. 2, inositol-induced currents

Fig. 3. The effect of JAK2 is reversed by the JAK2 inhibitor AG490. A. Representative original tracings showing inositol (1 mM) - induced current (I_{SMIT}) in *Xenopus* oocytes injected with water (a) or with cRNA encoding SMIT (b) or SMIT + JAK2 (c-e). Experiments were done in the absence of inhibitor (c) or following 8 hours (d) or 24 hours (e) pretreatment with JAK2 inhibitor AG490 (40 μ M). B. Arithmetic means $\pm$ SEM (n = 20) of inositol (1 mM) - induced current (I_{SMIT}) in *Xenopus* oocytes injected with water (H_2O , dotted bar), or expressing SMIT with JAK2 (black and grey bars) in the absence of inhibitor (black bar) or following pretreatment with the JAK2 inhibitor AG490 (40 μ M, light grey bars) for the indicated time periods. , ### indicates statistically significant (p<0.001) difference from the absence of JAK2 inhibitor AG490. *** indicates statistically significant (p<0.001) difference from SMIT alone (absence of JAK2).

Fig. 4. Effects of brefeldin A and actinomycin D on SMIT and SMIT + JAK2 expressing *Xenopus* oocytes. A. Arithmetic means $\pm$ SEM (n = 21) of inositol (1 mM) - induced current (I_{SMIT}) in *Xenopus* oocytes injected with cRNA encoding SMIT with JAK2 and incubated for the indicated time periods in the absence (black bars) and presence (light grey bars) of Brefeldin A (5 μ M). *, *** indicates statistically significant (p<0.05, p<0.001) difference from the absence of JAK2; #, ## indicates statistically significant (p<0.05, p<0.01) difference from the absence of brefeldin A. B. Arithmetic means $\pm$ SEM (n = 15) of inositol (1 mM) - induced current (I_{SMIT}) in *Xenopus* oocytes injected with cRNA encoding SMIT without (white bars) and with JAK2 (dark grey bars) in the presence and absence of 10 μ M actinomycin D 24-48 hour prior to the measurement. *** indicates statistically significant different (p<0.001) from the absence of JAK2.

in SMIT-expressing *Xenopus* oocytes increased following increase of inositol concentration. The currents allowed calculating a maximal current, which approached 6.30 ± 0.03 nA (n = 9) in *Xenopus* oocytes expressing SMIT alone, and 3.84 ± 0.02 (n = 9) in *Xenopus* oocytes expressing SMIT together with JAK2. Accordingly, the maximal transport rate was significantly lower in *Xenopus* oocytes expressing SMIT together with JAK2 than in *Xenopus* oocytes expressing SMIT alone. The inositol concentration required for halfmaximal current (K_M) approached 0.06 ± 0.01 μ M (n = 9) in *Xenopus* oocytes expressing SMIT alone, and 0.12

$\pm 0.04 \mu\text{M}$ ($n = 9$) in *Xenopus* oocytes expressing SMIT together with JAK2. The K_M was not significantly different between *Xenopus* oocytes expressing SMIT together with JAK2 and *Xenopus* oocytes expressing SMIT alone (Fig. 2).

Treatment of *Xenopus* oocytes expressing both, SMIT and JAK2, with the JAK2 inhibitor AG490 (40 μM) was followed by an increase of I_{SMIT} (Fig. 3). The effect of the inhibitor on I_{SMIT} reached statistical significance within 8 and 24 hours of preincubation with AG490.

The decrease of I_{SMIT} in SMIT expressing *Xenopus* oocytes following coexpression of JAK2 could have been due to accelerated clearance of carrier protein from the cell membrane. In order to estimate the stability of I_{SMIT} , the SMIT-expressing *Xenopus* oocytes were treated with 5 μM brefeldin A, a substance blocking the insertion of new carrier protein into the cell membrane. As shown in Fig. 4A, in the presence of brefeldin A I_{SMIT} declined within 8 and 24 hours to a similar extent in *Xenopus* oocytes expressing SMIT together with JAK2 and in *Xenopus* oocytes expressing SMIT alone. The observation suggests that JAK2 decreases I_{SMIT} by a mechanism other than accelerating carrier clearance from the cell membrane.

Inhibition of transcription by incubation (24-48 hours) with actinomycin D (10 μM) did not significantly modify I_{SMIT} , which remained significantly lower in *Xenopus* oocytes expressing SMIT together with JAK2 than in *Xenopus* oocytes expressing SMIT alone (Fig. 4B). Thus, the effect of JAK2 did not involve altered transcription.

Discussion

The present study discloses that JAK2 contributes to the regulation of the myoinositol transporter SMIT. Coexpression of the Janus Kinase 2 (JAK2) significantly decreased the electrogenic transport of inositol in SMIT expressing *Xenopus* oocytes. Down-regulation of SMIT was similarly observed following coexpression of the gain of function mutant V^{617F} JAK2 but not following coexpression of inactive K^{882E} JAK2. Thus, JAK2 is effective as kinase. Coexpression of the kinase significantly decreased the maximal current. Thus, JAK2 is at least partially effective by decreasing the maximal transport rate of the carrier. JAK2 expression did not significantly affect K_M . However, the large scatter of calculated K_M values precludes safe conclusions on alterations of substrate affinity.

JAK2 has previously been shown to foster cellular glucose uptake by influencing facilitative glucose carriers [49, 50] and Na^+ coupled glucose transport [51]. Moreover, JAK2 participates in the regulation of Na^+ coupled neutral amino acid transporter B(0)AT (SLC6A19) [52], Na^+ coupled glutamate transport [48] and Na^+ and Cl^- coupled transport of betaine and GABA [20].

Inhibition of carrier insertion into the cell membrane by brefeldin A leads to a decline of SMIT activity, which is similar in *Xenopus* oocytes expressing SMIT together with JAK2 and *Xenopus* oocytes expressing SMIT alone. Thus, the clearance of carrier from the cell membrane appears not to be sensitive to JAK2 expression. Alternatively, JAK2 may be effective by fostering carrier insertion into the cell membrane or by activating the carrier within the cell membrane.

Exposure of *Xenopus* oocytes expressing SMIT together with JAK2 to the JAK2 inhibitor AG490 was followed by a gradual increase of SMIT induced current indicating that the inhibitor counteracts the suppression of the carrier by the kinase. The effect appears to be slow, which may point to indirect regulation of the carrier. The observations in the presence of actinomycin D suggest that the observed effect is not due to an effect of JAK2 on gene transcription.

The presently observed down-regulation of SMIT by JAK2 may be considered surprising in view of the stimulating effect of hypertonicity on JAK2 [18, 19]. Osmotic cell shrinkage stimulates the expression of osmolyte transporters [53-55] including SMIT [21, 24-30, 35-40]. Along those lines, JAK2 increases the activity of the osmolyte transporter BGT1 [20] and Na^+/H^+ exchanger [56], which similarly serve to increase cell volume following osmotic cell shrinkage [57]. However, JAK2 is similarly activated by energy depletion [17] and the

kinase may inhibit SMIT to counteract cell swelling, a major threat of energy depleted cells [57].

In theory, the observed downregulation of SMIT by JAK2 may be relevant for the effects of JAK2 activating hormones and cytokines [1-3], such as leptin [4], growth hormone [5, 6], erythropoietin [3], thrombopoietin [3] and granulocyte colony-stimulating factor [3]. To the best of our knowledge, however, an effect of those hormones on SMIT expression and function has never been explored.

In conclusion, the present paper reveals that JAK2 is a powerful regulator of the Na⁺, Cl⁻ coupled myoinositol transporter SMIT. JAK2 down-regulates the transporter and thus limits the cellular uptake of Na⁺, Cl⁻ and myoinositol.

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