The suppressor of cytokine signalling 2 (SOCS2) is a key repressor of insulin secretion

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Abstract

Aims/hypothesis Suppressor of cytokine signalling (SOCS) proteins are powerful inhibitors of pathways involved in survival and function of pancreatic beta cells. Whereas SOCS1 and SOCS3 have been involved in immune and inflammatory processes, respectively, in beta cells, nothing is known about SOCS2 implication in the pancreas.

Methods Transgenic (tg) mice were generated that constitutively produced SOCS2 in beta cells (β SOCS2) to define whether this protein is implicated in beta cell functioning and/or survival.

Results Constitutive production of SOCS2 in beta cells leads to hyperglycaemia and glucose intolerance. This phenotype is not a consequence of decreased beta cell mass or inhibition of insulin synthesis. However, insulin secretion to various secretagogues is profoundly altered in intact animals and isolated islets. Interestingly, constitutive SOCS2 production dampens the rise in cytosolic free calcium concentration induced by glucose, while glucose metabolism is unchanged. Moreover, tg islets have a depletion in endoplasmic reticulum Ca^{2+} stores, suggesting that SOCS2 interferes with calcium fluxes. Finally, in

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β SOCS2 mice proinsulin maturation is impaired, leading to an altered structure of insulin secretory granules and augmented levels of proinsulin. The latter is likely to be due to decreased production of prohormone convertase 1 (PC1/3), which plays a key role in proinsulin cleavage.

Conclusions/Interpretations SOCS2 was shown to be a potent regulator of proinsulin processing and insulin secretion in beta cells. While its constitutive production is insufficient to induce overt diabetes in this mouse model, it causes glucose intolerance. Thus, increased SOCS2 production could be an important event predisposing to beta cell failure.

Keywords Glucose intolerance · Insulin secretion · Pancreatic beta cell · Proinsulin maturation · SOCS · Transgenic mice

Abbreviations

CICR	Calcium-induced calcium release
CPE	Carboxypeptidase E/H
ER	Endoplasmic reticulum
GH	Growth hormone
HFD	High-fat diet
IPGTT/OGTT	Intraperitoneal glucose tolerance test/oral glucose tolerance test
JAK	Janus kinase
PC	Prohormone convertase
r-t PCR	Real-time PCR
RIP	rat insulin promoter
SERCA	Sarcoendoplasmic reticulum Ca^{2+} ATPase
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
tg	Transgenic
WT	Wild-type

Introduction

Beta cell dysfunction is fundamental in the development of both type 1 and type 2 diabetes. While it is accepted that autoimmune destruction of islets is responsible for type 1 diabetes, the mechanisms of pancreatic failure involved in deterioration of insulin resistance to type 2 diabetes remain to be clarified. Generally speaking, in the prediabetic obese state insulin resistance is coupled to compensatory hyperinsulinaemia, followed by beta cell failure and ensuing chronic hyperglycaemia characteristic of diabetes. As a

consequence of insulin resistance several perturbations occur, such as increased circulating levels of cytokines and metabolites, and they together participate in beta cell dysfunction [1–4].

The suppressor of cytokine signalling (SOCS) proteins have received much attention for their inhibitory role in signalling by cytokines [5]. During recent years, SOCS have emerged as potent modulators of additional pathways, such as those induced by insulin and growth hormone (GH) [6–10]. Concerning the pancreas, the role of SOCS proteins has so far been mainly studied in relation to the action of cytokines [11–13]. Among the different SOCS, SOCS3 has been the most intensively studied in pancreatic islets. Recent data from our laboratory demonstrate that in pancreatic beta cells SOCS3, induced by cytokines, interacts with the insulin receptor, inhibiting the recruitment of IRS proteins and hence downstream signals [14]. Several studies from Billestrup et al. reported that SOCS3 constitutively produced in beta cells reduces cytokine [15, 16] and GH [9, 17] signals in these cells. Indeed, the latter observations clearly demonstrate the important role of SOCS3 in inhibiting deleterious cytokine signalling in beta cells and hence favouring islet survival [15, 16]. In addition, using a mouse model producing SOCS3 constitutively in beta cells, Billestrup et al. revealed the implication of SOCS3 in the regulation of GH signalling in the endocrine pancreas [9].

Several studies illustrate the impact of SOCS2 on somatic growth through regulation of GH/IGF1 pathways [8, 18, 19]. In addition, SOCS2 plays a role in the nervous system [20–22] and the immune response [23] and might participate in certain cancers [24–26]. To date, little is known about the impact of SOCS2 on pancreatic physiology. However, it has been recently shown that SOCS2 single nucleotide polymorphisms (SNPs) can be associated with type 2 diabetes [27]. Moreover, the authors found that constitutive production of SOCS2 in MIN6 beta cells reduces glucose-stimulated insulin secretion. Several genome-wide analyses have recently revealed multiple genetic loci specifically associated with diabetes [28, 29]. It is intriguing that the products of most of those genes are potentially involved in beta cell function, suggesting that beta cells are chief players in the pathogenesis of type 2 diabetes, and that predisposition to this disease is likely to be related to inappropriate expression of specific beta cell genes. Given the role of SOCS2 in the regulation of growth and cytokine signals, we put forward the idea that SOCS2 could play a key role in beta cell function. To test our view, we generated a mouse model producing SOCS2 constitutively in beta cells, and studied the ability of these mice to maintain glucose homeostasis.

Methods

Generation of transgenic mice producing SOCS2 protein in beta cells

The vector containing the rat insulin promoter (RIP) 1 is a gift from B. Thorens (University of Lausanne, Lausanne, Switzerland), and has been previously described [30]. Briefly, a flag-tagged complete coding sequence of mouse *Socs2* cDNA (gift from D. Hilton, The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia) [31] was introduced in pXf3-RIP1. Our mice had a C57BL/6 background (Elevage R. Janvier, Le Genest St Isle, France). Care and use of mice was in accordance with the Guidelines of the French National Institute of Health and Medical Research (INSERM, France).

Animal studies and metabolic analysis

Mice were housed on a 12 h light/dark cycle and put when 6 weeks old on a high-fat diet (HFD) (42% fat, TD-88137; Harlan Teklab, Madison, WI, USA) or standard chow diet with free access to diet and water. Body weight was measured weekly. Blood glucose was measured using the One Touch glucometer system (Lifescan Inc., Milpitas, CA, USA), serum insulin using Ultra Sensitive Rat Insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA), and proinsulin using Rat/Mouse Proinsulin ELISA kit (Mercodia AB, Uppsala, Sweden). For intraperitoneal and oral glucose tolerance tests (IPGTT and OGTT, respectively) 2 mg/g body weight of glucose was given. For glucose- and arginine-stimulated insulin secretion, animals were starved overnight and injected intraperitoneally with glucose (2 mg/g) or arginine (3 mg/g).

Pancreatic extracts and insulin content

Insulin was extracted from whole pancreas with acid/alcohol as previously described [32] and hormone content was measured using ELISA assay (Mercodia AB).

RNA extraction, reverse transcription and real-time PCR (r-t PCR)

Isolated islets were homogenised in Trizol (Invitrogen Life Technologies Inc., Gaithersburg, MD, USA), RNAs were extracted and reverse-transcribed (High capacity, Applied Biosystems Inc., Foster City, CA, USA). cDNAs were analysed using SYBR Green r-t PCR (ABI PRISM 7000 Sequence Detector System). The amount of cDNA used in each reaction was normalised to housekeeping *36b4* (also known as *Rplp0*) cDNA.

Protein extraction and western blotting

For protein extraction, isolated islets were lysed in RIPA buffer and proteins were quantified (BCA protein assay kit; Pierce, Thermo Fisher Scientific, Rochford, IL, USA), separated by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes and blotted with antibodies. Immunoreactive proteins were revealed by enhanced chemiluminescence (Millipore, Billerica, MA, USA). Antibodies to SOCS2 were from Cell Signaling Technology, and to tubulin from Sigma.

Insulin secretion by isolated islets

Islets were isolated according to Kulkarni et al. [33]. Following isolation, islets were incubated overnight in RPMI medium (7 mmol/l glucose; 0.2% [wt/vol.] BSA). Next, islets were deprived in KRBH medium (1 mmol/l glucose for 45 min) and stimulated with various secretagogues in the presence of 2.8 mmol/l glucose. Islet supernatant fraction was collected after 1 h of treatment and insulin was extracted from islets with acid/alcohol solution (1.5%/75%).

Intracellular Ca^{2+} concentration measurements

Islets were incubated with fura-2 acetoxymethyl ester (final concentration 2 $\mu\text{mol/l}$) for 60 min at 37°C in a Krebs–Ringer bicarbonate-buffered solution containing 2.8 mmol/l glucose. The coverslips were then analysed by epifluorescence (Diaphot TDM, Nikon). Fura-2 fluorescence of single islets was measured by dual excitation fluorimetry using a camera-based image analysis system (Metafluor, Universal Imaging). Data are presented as the ratios of the 340 and 380 nm signals as described [34].

Measurement of glucose metabolism

The methods to measure D-[5- ^3H]glucose metabolism and D-[U- ^{14}C]glucose oxidation in islets are described [35–37].

Histological analysis

For transmission electron microscopic analysis pancreases were fixed in 2.5% (vol./vol.) glutaraldehyde and 2% (vol./vol.) paraformaldehyde, post-fixed in 1% (vol./vol.) osmium tetroxide, stained in aqueous uranyl acetate, dehydrated and embedded in epoxy resin. Ultrathin sections (70 nm) were stained using lead citrate and examined by transmission electron microscopy (Jeol 1400; Hitachi).

For immunohistochemistry, the following antibodies were used: guinea pig anti-insulin antibody (1:100; Sigma,

St Louis, MO, USA); rabbit anti-mouse SOCS-2 antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse anti-human PC1 antibody (1:50; Abd Serotec, Raleigh, NC, USA); anti-rabbit Alexa Fluor 488 (1:100; Invitrogen); anti-guinea pig Alexa Fluor 568 (1:100; Invitrogen); anti-mouse IgG biotinylated (1:250; Vector Laboratories, Ontario, LTN, Canada); and streptavidin Alexa Fluor 488 (1:100; Molecular Probes). To-Pro-3 iodide (Molecular Probes, Invitrogen) for nuclear counterstaining was used for laser-scanning confocal analysis (TCS SP2; Leica Microsystems).

Morphometry

Immunohistochemistry was performed on consecutive sections (7 μ m) of paraffin-embedded pancreatic tissue [38]. Insulin-stained sections were used for morphometric analysis. Quantitative evaluation was performed using the Histolab 6.0.5 software (Microvision Instruments). Beta cell mass was calculated by multiplying the relative beta cell area by pancreas weight. Slides were counterstained with haematoxylin.

Statistical analysis

Results are presented as means $\pm$ SEM. *n* represents the number of mice. Differences between groups were compared with the two-tailed unpaired Student's *t* test. A *p* value of <0.05 was considered significant.

Results

Constitutive production of SOCS2 in beta cells

To study the role of SOCS2 in beta cells, we generated transgenic mice constitutively producing SOCS2 specifically in beta cells. Note that normally the SOCS2 protein is present at very low levels in the basal condition. Two separate transgenic sublines were analysed. Our transgenic mice had normal fertility and survival, and had a Mendelian transgene distribution. Since the same phenotype was obtained for males and females, we show only results observed with males. We verified the constitutive expression of *Socs2* in pancreatic islets by r-t PCR (Fig. 1a) and

Fig. 1 Generation of transgenic mice constitutively producing SOCS2 in beta cells. **a** Constitutive *Socs2* transgene expression. Islets were isolated from transgenic (β SOCS2, black bar) and wild-type (WT, white bar) mice ($n=10$ in each group). RNA was extracted and analysed by r-t PCR. **b** Total extracts from 200 isolated islets from β SOCS2 and WT mice were prepared and analysed by western blot with antibody to SOCS2. Antibody to tubulin was used as a control. **c, d** Total pancreas was isolated from tg or WT mice ($n=3$), fixed and embedded in paraffin. Immunohistochemistry was performed with antibodies to SOCS2 or to insulin. Representative images show the restrictive production of SOCS2 in β SOCS2 mice and in insulin-producing cells. Scale bar, 20.25 μ m

western blotting (Fig. 1b). Moreover, by immunohistochemistry we demonstrated that SOCS2 is detected exclusively in β SOCS2 pancreatic islets (Fig. 1c), and that this signal corresponded exactly with insulin staining (Fig. 1d).

Phenotypic analysis of β SOCS2 mice

Body weight Animals on chow or high-fat diet were weighed every week. As shown in Fig. 2a, no difference

Fig. 2 Metabolic study of β SOCS2 transgenic mice. **a** β SOCS2 mice have a body weight similar to WT. Mice on chow diet (WT, $n=15$, regular discontinuous line; β SOCS2, $n=15$, regular continuous line) or on HFD (WT, $n=15$, bold discontinuous line; β SOCS2, $n=15$, bold continuous line) were weighed once a week starting at 6 weeks of age and for 11 weeks. **b**, **c** β SOCS2 mice are hyperglycaemic but only slightly hypoinsulinaemic. Glycaemia (**b**) and insulinemia (**c**) were measured in the serum of 12-week-old mice under overnight fasted or fed conditions. Four groups were analysed: WT mice on chow diet ($n=10$; white bars), β SOCS2 mice on chow diet ($n=8$; black bars), WT mice on HFD ($n=5$; grey bars), and β SOCS2 mice on HFD ($n=5$; black and white bars). * $p<0.05$. **d** Glycaemia was measured in the serum of 8–21-week-old mice in overnight fasted conditions. Same groups as described in **b**, **c**. Hyperglycaemia of tg mice is significant in all conditions (* $p<0.05$)

in body weight was observed for transgenic (tg) compared with wild-type (WT) mice on the two diets.

Serum variables Glycaemia and insulinaemia were measured at different ages. Results obtained with 12-week-old mice are presented in Fig. 2b, c. Blood glucose levels in β SOCS2 mice were significantly higher than in WT mice, and this pattern is exacerbated on a high-fat diet (Fig. 2b). Concerning insulinaemia, β SOCS2 mice on a chow diet are hypoinsulinaemic in fasted conditions compared with WT mice, in correlation with their slight hyperglycaemia (Fig. 2c). However, we did not observe such hypoinsulinaemia in fed conditions with chow or high-fat diet. Hyperglycaemia in β SOCS2 mice only slightly increased with age (Fig. 2d).

Glucose tolerance As β SOCS2 mice were hyperglycaemic compared with WT mice, we analysed their glucose tolerance (Fig. 3a, b). β SOCS2 transgenic mice on chow and high-fat diet clearly suffered from glucose intolerance when exposed to a glucose challenge by either intraperitoneal (Fig. 3a) or oral (Fig. 3b) administration. The difference between tg and WT animals was significant at all time points studied. While the glucose intolerance of tg mice was already detectable on the chow diet, it was exacerbated on the high-fat diet. The glucose tolerance tests shown were performed on 12-week-old animals. To summarise, β SOCS2 animals suffer from severe glucose intolerance, which is consistent with their hyperglycaemia. Our phenotypic analysis reveals that β SOCS2 mice are severely glucose-intolerant. In addition, insulin tolerance tests did not reveal insulin resistance in β SOCS2 mice (data not shown). Further, no pronounced worsening of the phenotype, i.e. development of overt diabetes, was observed on a high-fat diet. Considering the hallmarks of the phenotype of our mice, we favoured the idea that their hyperglycaemia was due to insufficient insulin secretion rather than to a dysfunction of peripheral tissues.

Insulin secretion To evaluate the insulin secretory response of β SOCS2 mice compared with WT mice, we analysed insulin secretion induced by glucose and arginine in 15-week-old mice. Secretagogues were injected intraperitoneally and insulinaemia was measured from 2 to 30 min after injection. As previously described, glucose-stimulated insulin secretion is biphasic in WT animals (Fig. 3c), i.e. a rapid and transient spike at 2 min, followed by a second phase from 10 to 30 min. Remarkably, in tg mice the first secretion peak is completely abolished, while the second phase is severely dampened. This secretory defect was confirmed using arginine, which in the presence of low glucose induces a main secretion spike at 2 min (Fig. 3d).

Fig. 3 β SOCS2 mice are glucose-intolerant. **a**, **b** GTTs in β SOCS2 transgenic mice on chow (regular continuous line) and HFD (bold continuous line), and WT on chow (regular discontinuous line) and HFD (bold discontinuous line). Intraperitoneal IPGTTs (**a**) ($n=10$ in each group) and OGTTs (**b**) ($n=5$ in each group) were performed on 15-week-old mice after a 16 h fast (overnight), and glucose was given (2 mg/g) intraperitoneally and orally, respectively. **c**, **d** Insulin

secretion in intact animals (15 weeks old). Overnight starved mice were challenged with glucose (2 mg/g) (**c**) or arginine (3 mg/g) (**d**), and serum was collected at different time points after intraperitoneal injection. Circulating insulin levels were quantified using an insulin ELISA kit (WT, discontinuous line, $n=15$; β SOCS2, continuous line, $n=15$). $*p<0.05$

Fig. 4 **a**, **b** Morphometric analysis. Pancreases were extracted from WT and β SOCS2 mice (15–20 weeks old), fixed in paraformaldehyde and embedded in paraffin. Pancreatic sections 7 μm thick were stained with antibody to insulin and counterstained with haematoxylin/eosin (H/E). **a** A representative image of H/E staining is shown; WT on the left and β SOCS2 on the right. **b** The beta cell mass was calculated by multiplying the percentage of insulin-positive area by the pancreas weight ($n=4$ for each group). Quantitative evaluation was performed

using Histolab 6.0.5 software (WT, white bar; β SOCS2, black bar). **c** Insulin mRNA expression. Total RNA was extracted from isolated islets (WT, white bar, $n=10$; β SOCS2, black bar, $n=10$) mice (10–15 weeks old), reverse transcribed and analysed by SYBR Green r-t PCR using insulin-specific primers. **d** Insulin was extracted from whole pancreas with acid/alcohol solution and quantified by ELISA (WT, white bar, $n=3$; β SOCS2, black bar, $n=3$). Scale bar, 40 μm

Islet biology of β SOCS2 mice

Morphometry To search for defects in the pancreas of β SOCS2 mice that could explain their insulin secretory defect, we performed islet morphometry. Results of a representative experiment are shown in Fig. 4a, b. No significant variation was found in β SOCS2 mice compared with WT mice. The beta cell mass of mice fed with a high-fat diet was, as expected, enlarged in WT mice, and this was found to a similar extent with tg mice (data not shown). To sum up, the altered insulin secretion in β SOCS2 mice is unlikely to be due to a reduction in beta cell mass.

Insulin mRNA expression and content Using r-t PCR, we found that the insulin mRNA levels were similar in tg

and WT animals (Fig. 4c). A similar pattern was obtained for insulin content in whole pancreas (Fig. 4d). This suggests that the defect in β SOCS2 mice may concern insulin maturation and/or secretion rather than its production.

Beta cell function in isolated islets

Insulin secretion To investigate whether the defective insulin secretion seen in β SOCS2 mice was generated in vivo by noxious environmental conditions or was the consequence of an autonomous islet dysfunction, we looked at the secretory response of isolated islets induced by several secretagogues. We took advantage of stimuli such as glucose, KCl, arginine and glibenclamide, which

Fig. 5 **a** Insulin secretion in isolated islets. Isolated islets from WT ($n=5-9$) and β SOCS2 ($n=5-9$) were exposed to 2.8 mmol/l glucose (white bars), 20 mmol/l glucose (black bars), arginine 20 mmol/l (light grey bars), KCl 30 mmol/l (dark grey bars) and glibenclamide 1 μ mol/l (black and white bars). Non-glucose secretagogues were tested in the presence of 2.8 mmol/l glucose. Insulin levels were quantified in the supernatant fraction of isolated islets 1 h after stimulation and normalised to intra-islet insulin content. Results are expressed as a multiple of 2.8 mmol/l glucose condition. **b-d** Cytosolic free Ca^{2+} concentration in response to 16.7 mmol/l glucose (**b**), 50 mmol/l KCl in the presence of 5.6 mmol/l glucose (**c**) and 50 mmol/l KCl without

glucose (**d**). Experiments were performed on perfused islets from WT ($n=4$, discontinuous line) and β SOCS2 ($n=4$, continuous line). **e,f** Glucose oxidation and ^3H_2O production were measured in isolated islets (WT, $n=3$; β SOCS2, $n=4$) in the presence of 2.8 (white bars) and 16.7 mmol/l glucose (black bars). **g** Estimation of the calcium content in the endoplasmic reticulum (ER). $[Ca^{2+}]_i$ was measured in the presence of 2 μ mol/l thapsigargin. Increase in $[Ca^{2+}]_i$ corresponds to a calcium leakage from ER in WT ($n=4$, discontinuous line) and β SOCS2 ($n=4$, continuous line) isolated islets. Representative of four individual experiments in each case. * p < 0.05. NS, not significant

are known to activate the secretory machinery by different means. Interestingly, we found that insulin secretion in response to the tested secretagogues was significantly reduced in β SOCS2 islets compared with WT (Fig. 5a). These observations confirmed the results obtained in vivo, and suggest that the defect is likely to occur at some common distal step in the secretory machinery.

Cytosolic free calcium concentration ($[Ca^{2+}]_i$) Here we determined the $[Ca^{2+}]_i$ in β SOCS2 and WT islets in response to glucose and KCl. As shown in Fig. 5b, SOCS islets showed a delayed and reduced initial phase. Thus, the increase in $[Ca^{2+}]_i$ over the baseline value (0–3 min) recorded during the first phase (3–10 min) was reduced by 45% ($p<0.05$). In the presence of low glucose (5.6 mmol/l), KCl-induced $[Ca^{2+}]_i$ increase over the baseline value (0–2 min) was reduced by 21% ($p<0.05$) in β SOCS2 islets (Fig. 5c). However, in the absence of glucose, the increase in $[Ca^{2+}]_i$ in response to KCl is similar in WT and β SOCS2 islets (Fig. 5d), suggesting that glucose metabolism may be altered in transgenic mice.

Glucose metabolism Next we measured glucose oxidation and ^3H_2O production reflecting the complete process of glucose metabolism (Fig. 5e, f). Our analysis revealed unaltered glucose metabolism in islets from β SOCS2 compared with tg mice, and this corresponds with the unchanged levels of insulin mRNA. Thus, the perturbation leading to the insulin secretion defect in β SOCS2 mice concerns the regulation of $[Ca^{2+}]_i$, but is unlikely to be related to glucose metabolism and plasma membrane depolarisation.

Endoplasmic reticulum calcium ($[Ca^{2+}]_{ER}$) content It is well documented that calcium contained in the endoplasmic reticulum (ER) participates in the regulation of $[Ca^{2+}]_i$ in beta cells. In particular, Ca^{2+} release from ER in response to extracellular Ca^{2+} entrance (called CICR, for Ca^{2+} -induced Ca^{2+} release) may be implicated in controlling insulin secretion. Therefore, we estimated $[Ca^{2+}]_{ER}$ by quantifying $[Ca^{2+}]_i$ in the presence of thapsigargin, which blocks the capture of Ca^{2+} from cytosol to ER lumen by inhibiting the action of SERCAs. As expected, in WT islets, an increase in $[Ca^{2+}]_i$ resulting from the leak of Ca^{2+} from ER was observed. Interestingly, this effect (5–20 min) was reduced by 48% ($p<0.01$) in islets from tg mice (Fig. 5g). A similar result was observed in the presence of cyclopiazonic acid (data not shown).

In conclusion, the secretory defect due to constitutive production of SOCS2 in beta cells is probably operating independently of plasma membrane depolarisation, and is due, at least partially, to a decrease in intracellular Ca^{2+} stores.

Fig. 6 **a** Ultrastructural analysis of islets. Pancreases from 15-week-old WT ($n=3$) and β SOCS2 ($n=3$) mice were fixed in glutaraldehyde/PFA and embedded in epoxy resin. Ultrathin sections stained with lead citrate were examined by transmission electronic microscopy. Representative pictures are shown. Black scale bar=2 μ m. **b** Expression of genes coding for enzymes involved in insulin maturation. Total RNA was extracted from isolated islets (WT, white bar, $n=6$; β SOCS2, black bar, $n=5$), reverse transcribed and analysed by SYBR Green real-time PCR using primers specific to *Pc-1*, *Pc-2* and *Cpe*. **c** PC1 protein content in WT and β SOCS2 mice ($n=3$ for each group, 15 weeks old). Representative images of immunohistochemistry analysis using a specific PC1 antibody are shown. * $p<0.05$. Scale bar, 53.66 μ m

Insulin maturation and exocytosis

Insulin granule appearance Electron microscopic analysis revealed in β SOCS2 beta cells an altered structure of the insulin secretory granules (Fig. 6a). Whereas insulin granules are abundant in WT mice and contain a classical dense core, in tg mice they are smaller and do not appear as mature granules. This perturbed granule pattern in tg animals could reflect a defect in proinsulin maturation.

mRNA levels of enzymes implicated in proinsulin maturation The insulin packaged in mature granules derives from proinsulin, which is cleaved inside the secretory granules by several enzymes, including pro-hormone convertase 1/3 (PC1), pro-hormone convertase 2 (PC2) and carboxypeptidase E/H (CPE). In trying to explain the altered structure of insulin granules in tg mice, we analysed by r-t PCR the mRNA levels of these key enzymes. In tg islets we found a 50% decrease in *Pc1* mRNA levels (Fig. 6b), but unchanged *Pc2* and *Cpe* mRNA levels.

PC1 protein levels in β SOCS2 mice Next, we investigated whether, in accordance with the r-t PCR results, the PC1 protein levels are decreased in tg mice. As shown in Fig. 6c, immunohistochemistry staining of PC1 is severely impaired in islets from β SOCS2 mice compared with WT.

To conclude, constitutive production of SOCS2 in beta cells could perturb the proinsulin maturation by affecting the appropriate levels of the key prohormone convertase PC1.

Circulating levels of proinsulin in β SOCS2 mice compared with WT

To confirm the fact that β SOCS2 mice have perturbed proinsulin maturation, we quantified by a specific ELISA

test the levels of circulating proinsulin. Interestingly, the proinsulin content was increased in sera of tg animals compared with WT (Fig. 7a). This pattern was observed for all the conditions tested (fasted/fed; chow/HFD). In addition, by normalising the circulating proinsulin levels to those of insulin (shown in Fig. 2c), we showed that the ratio of proinsulin/total insulin is robustly increased in tg animals (Fig. 7b). Thus, in WT mice 18.3% proinsulin and 81.7% mature insulin were found, while in tg mice 58% proinsulin and 42% mature insulin were present. This shift in favour of proinsulin was observed on both chow and a high-fat diet.

Discussion

Here we approached the role of SOCS2 in pancreatic beta cell functioning. To this end, we generated β SOCS2 transgenic mice constitutively producing SOCS2 protein specifically in beta cells. Remarkably, starting from 8–10 weeks the β SOCS2 mice were overtly hyperglycaemic and glucose-intolerant. This phenotype, observed in two separate tg sublines (ESM Fig. 1), was not profoundly worsened with age or with a high-fat diet, and the tg mice do not develop overt diabetes. A key feature of our mice is their altered insulin secretion in vivo. Counterintuitively, this was not due to a reduced beta cell mass, which was in fact even slightly augmented in β SOCS2 mice compared with WT mice. It is known that GH plays a key role in the regulation of beta cell mass [39] and that SOCS2 is a potent inhibitor of GH signalling. However, our data suggest that GH signalling was probably not affected by SOCS2, since no islet hypoplasia was detected. Our result correlates with observations made with genetically modified animals in which constitutive production of SOCS2 does not necessarily inhibit GH signalling [40, 41]. In addition, Billestrup et al. showed a reduced beta cell mass in mice constitu-

Fig. 7 a Circulating proinsulin levels. Using a specific ELISA kit, we measured proinsulin levels in the serum of 12-week-old WT and β SOCS2 mice on chow or HFD and in fasted or fed conditions (WT chow diet, white bars, $n=10$; β SOCS2 chow diet, black bars, $n=8$; WT HFD, grey bars, $n=5$; β SOCS2 HFD, black and white bars, $n=3$).

b Ratio of proinsulin to insulin in the serum of WT and β SOCS2 mice. Proinsulin levels (grey) were expressed as a percentage of total insulin levels. Mature insulin levels (black) were estimated as (100% - % proinsulin). * $p<0.05$

tively producing SOCS3 in these cells. Together, these results suggest that in beta cells SOCS3 is more potent than SOCS2 to inhibit GH signalling. Using morphometric analysis, we found that high-fat diet-induced hyperplasia of beta cell mass occurs in tg as well as in WT mice. This suggests that the molecular events involved in compensatory islet hyperplasia, thought to be largely dependent on insulin action [42], were not perturbed by SOCS2 constitutive production. Taken together, our data would indicate that SOCS2 does not affect beta cell proliferation and/or replication, but, rather, beta cell functioning.

Indeed, it appears that constitutive SOCS2 production in beta cells leads to reduced insulin secretion in response to several secretagogues (glucose, KCl, arginine and glibenclamide). This defect is likely to be intrinsic to beta cells, as it is also observed with isolated islets. The molecular mechanisms implicated in insulin secretion are well known [43]. In brief, after its uptake extracellular glucose is metabolised, generating ATP. The increased ATP/ADP ratio leads to potassium channel closure, plasma membrane depolarisation and opening of voltage-dependent Ca^{2+} channels. The rise in intracytoplasmic Ca^{2+} drives exocytosis of mature insulin contained in secretory granules. In our βSOCS2 mice the insulin secretion defect appears to occur at a major insulin secretion-coupling step, since it is found with different types of secretagogues. Looking at distal molecular steps implicated in insulin secretion, we showed in islets from tg mice that the glucose-induced increase in $[\text{Ca}^{2+}]_i$ is delayed and reduced, and that the potassium-induced increase in the presence of low glucose has a perturbed profile. However, in the absence of glucose, KCl, which increases $[\text{Ca}^{2+}]_i$ mainly by Ca^{2+} entry from the extracellular compartment, gave a similar $[\text{Ca}^{2+}]_i$ profile in tg and WT mice. While this could suggest that the defective insulin secretion in tg was linked to decreased glucose metabolism, we found this not to be the case. Thus, molecular events unrelated to glucose metabolism, but implicated in calcium fluxes, may be altered in tg islets, leading to impaired insulin secretion. Interestingly, we found that ER Ca^{2+} stores are decreased in islets from tg mice. The ER serves multiple important functions, including post-translational protein modification and folding, assembly of secretory proteins and Ca^{2+} storage. In particular, the ER participates in the control of $[\text{Ca}^{2+}]_i$ by the release of calcium from its intrinsic store (CICR) and by recapturing Ca^{2+} from the cytosol into its lumen. Calcium depletion from the ER lumen perturbs ER function and eventually beta cell survival [44]. Thus, the decrease in calcium contents in both cytosol and ER in our tg model corresponds with its insulin secretion defect. However, how SOCS2 alters Ca^{2+} fluxes in beta cells remains to be defined. Due to the high sensitivity of beta cells to ER stress, we investigated whether constitutive SOCS2 production in islets

would induce ER stress, shutting down beta cell function. As demonstrated in ESM Fig. 2, no increase in C/EBP homologous protein (CHOP) levels or inhibition of X-box binding protein-1 (XBP-1) splicing was detected in βSOCS2 tg islets. Moreover, the levels of SERCA2 protein and mRNA are unaltered in SOCS2-overproducing islets (ESM Figs 2 and 3). Thus, the ability of SOCS2 to deplete ER of calcium is independent of SERCA2 downregulation and does not lead to detectable ER stress.

In addition to its crucial role in insulin exocytosis [45], $[\text{Ca}^{2+}]_i$ favours the maturation of proinsulin to insulin inside the granules extruded from the distal endoplasmic reticulum [46, 47]. $[\text{Ca}^{2+}]_{\text{ER}}$ is also fundamental for processing enzymes implicated in the conversion of proinsulin to insulin (such as PC1/3 and PC2) and their inclusion inside the secretory granules. In accordance with these data, insulin granules in βSOCS2 islets appear as immature granules with reduced ‘condensed’ material. The secretory granules contain the enzymes necessary for cleavage of proinsulin to insulin plus C-peptide, i.e. PC-1/3, PC2 and CPE [48]. Remarkably, we found that PC-1/3 production was reduced at the mRNA and protein level in islets constitutively producing SOCS2. Importantly, PC-1/3 is the main enzyme implicated in proinsulin cleavage. Steiner et al. showed that the defect of proinsulin processing is more pronounced in PC-1/3 null mice than in PC-2 null mice [49, 50]. Indeed, in PC-1/3 null mice proinsulin and intermediate molecules represent 85% of total immunoreactive insulin, whereas they represent only 5.3% in WT mice. Interestingly, the heterozygous PC-1/3 null mice also exhibit altered proinsulin/insulin ratio and suffer from glucose intolerance. This correlates with our results demonstrating that a reduction by approximately half of *Pc1/3* mRNA expression, with unchanged *Pc-2* and *Cpe* levels, is sufficient to alter proinsulin maturation and glucose tolerance. Finally, we found that in our tg mice the level of circulating proinsulin is robustly increased. In correlation with this fact, the proinsulin contained in whole pancreas is significantly increased in βSOCS2 mice (ESM Fig. 4). Thus, as shown for the PC-1/3 null mice, the ratio of proinsulin/mature insulin is strongly augmented. However, how constitutive SOCS2 production leads to PC-1/3 downregulation represents a provocative question. It is interesting to remember that inflammatory stimuli can lead to activation of PC-1 production [51]. Indeed, in neuronal cells leukaemia inhibitory factor (LIF), IL6 or lipopolysaccharide (LPS) treatment is associated with increased *Pc1* mRNA level. It is well established that these stimuli are able to induce SOCS protein production, and that SOCS inhibit the signal that has led to their production. Moreover, LIF, LPS and IL6 mediate their intracellular actions through the gp130 receptor and the Jak/STAT pathway. As previously mentioned, SOCS proteins are potent negative

modulators of Jak/STAT signalling. While our most urgent challenge is to understand how SOCS proteins can affect expression of genes involved in proinsulin processing, our observations are particularly intriguing in light of recent reports showing that the confirmed type 2 diabetes-related genetic loci, TCF7L2, CDKAL1 and SLC30A8, are associated with impaired proinsulin conversion [52, 53].

To summarise, we report that constitutive production of SOCS2 in beta cells alters intracellular Ca^{2+} stores and is associated with decreased proinsulin maturation and impaired hormone secretion. This leads to pronounced glucose intolerance, but is insufficient to induce per se the development of overt diabetes. Our data reveal that a major actor in regulation of signalling by cytokines and hormones in beta cells, such as SOCS2, can impact on proinsulin processing and insulin secretion, and hence participate in beta cell failure, predisposing to diabetes.

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