Suppressor of cytokine signaling 1 (SOCS1) is an intracellular inhibitor of cytokine, growth factor, and hormone signaling. Socs1−/− mice die before weaning from a multiorgan inflammatory disease. Neonatal Socs1−/− mice display severe hypoglycemia and hypoinsulinemia. Concurrent interferonγ gene deletion (Ifng−/−) prevented inflammation and corrected the hypoglycemia. In hyperinsulinemic clamp studies, however, Socs1−/− mice had enhanced hepatic insulin sensitivity demonstrated by greater suppression of endogenous glucose production compared with controls with no difference in glucose disposal. Socs1−/−Ifng−/− mice had elevated liver insulin receptor substrate 2 expression (IRS-2) and IRS-2 tyrosine phosphorylation. This was associated with lower phosphoenolpyruvate carboxykinase mRNA expression. These effects were not associated with elevated hepatic AMP-activated protein kinase activity. Hepatic insulin sensitivity and IRS-2 levels play central roles in the pathogenesis of type 2 diabetes. Socs1 deficiency increases IRS-2 expression and enhances hepatic insulin sensitivity in vivo indicating that inhibition of SOCS1 may be a logical strategy in type 2 diabetes.

The suppressors of cytokine signaling (SOCS) are a family of proteins studied intensively because of their role in negative regulation of cytokine action through JAK/STAT signaling, but recent investigations have broadened the role of some SOCS family members including SOCS1, -2, -3, and -6 to regulation of inflammatory disease affecting the liver, pancreas, and heart. They die within 3 weeks of birth most likely from severe liver damage. It is therefore difficult to study metabolic effects of Socs1 deficiency in vivo in these mice, and other approaches have been adopted, potentially not resulting in complete Socs1 deficiency, including antisense oligonucleotide delivery in vivo. We have carried out metabolic studies in Socs1−/− mice protected from lethal inflammation by concurrent Ifng gene deletion that allowed Socs1−/− mice to be studied as adults. Neonatal Socs1−/− mice displayed severe hypoglycemia and hypoinsulinemia. We found that adult Socs1−/−Ifng−/− mice have metabolic abnormalities consistent with enhanced insulin sensitivity.

MATERIALS AND METHODS

Mice—Socs1−/− and Socs1−/−Ifng−/− mice have been described previously (11, 12). Experiments were initially performed on mice that were maintained on a mixed C57BL/6–129Sv genetic background. These were subsequently backcrossed to C57BL/6 for 10 generations, and experiments were then repeated on these mice. They were bred and housed in clean conditions in the animal facilities of the Walter and Eliza Hall Institute of Medical Research and St. Vincent’s Institute. Anti-IFNγ antibody treatment was as described previously (13). All procedures were approved by the animal ethics committee of the institutions involved.

Glucose and Insulin Measurements—Blood glucose levels were measured using Advantage II Glucose Strips with Advantage glucometer (Roche Diagnostics). Serum or plasma glucose concentrations were measured using a glucose analyzer (YSI, Inc., Yellow Springs, OH).
Serum or plasma insulin levels were measured by radioimmunoassay (Linco Research, St. Charles, MO).

**Metabolic Tests**—After an overnight fast, animals were anesthetized with 100 mg/kg pentobarbitone sodium (Nembutal, Rhone Merieux, QLD Australia). Catheters were inserted into the right jugular vein for infusion and the left carotid artery for plasma sampling. A tracheotomy was also performed to prevent airway obstruction during the experiment. Anesthesia was maintained by intravenous administration of pentobarbitone sodium, and body temperature was kept at 37 °C with a heating lamp.

Whole body glucose turnover was measured using the hyperinsulinemic-euglycemic clamp with continuous infusion (0.15 μCi/min) of [6-3H]glucose as described elsewhere (14, 15). During the clamp, insulin was infused at a constant rate of 1.5 milliunits/kg/min. Euglycemia was maintained by the infusion of 1% glucose in saline. Plasma samples were collected during steady state conditions at 90, 100, and 110 min. Under steady state condition, the rate of glucose appearance equals the rate of glucose disappearance. The rate of glucose appearance (Rg) was calculated by dividing the infusion rate of [6-3H]glucose (dpm/min) by the plasma [6-3H]glucose specific activity. The rate of endogenous glucose production was measured as the difference between the calculated Rg and the glucose infusion rate.

Glucose and arginine tolerance testing was performed as described previously (16). Briefly, after overnight fasting, animals were anesthetized and a catheter inserted into the left carotid artery for infusion and plasma sampling. Animals were allowed to recover from the surgery for 20 min. During the experiment body temperature was maintained at 37 °C with a heating lamp. A bolus of glucose (1g/kg) or arginine (1g/kg) was infused through the catheter, and blood samples were collected at 0, 2, 5, 10, 15, and 30 min from the catheter at regular intervals. The plasma was then assayed for glucose and insulin concentrations.

**Real Time PCR**—Tissues were collected from 12-week-old mice in a random fed (basal) state or after clamps and immediately frozen in liquid nitrogen. Tissues were mechanically homogenized (Ultra-Turrax T8, IKA, Staufen, Germany), and RNA was extracted according to the manufacturer’s directions (RNAwiz, Ambion, Austin, TX). Reverse transcriptase reaction was performed using AMV Reverse Transcriptase and Random Primers (Promega). The resultant cDNA was amplified using Rotorgene 3000 (Corbett Research, Sydney, Australia) with Assay-on-demand primers and FAM-labeled probes for SOCS3, phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase and 18S (Applied Biosystems, Foster City, CA). cDNA content in individual samples was normalized using 18S as a housekeeping gene.

**Measurement of Kinase Levels and Activity**—Liver extracts were homogenized in ice-cold lysis buffer and centrifuged at 14,000 × g for 50 min. Supernatants were removed, and the protein content was measured via the bicinchoninic acid method (Pierce). AMP-activated protein kinase (AMPK) activities were measured, in livers from fed mice, in immunocomplex from 500 μg of protein using AMPK α1 and α2 antibodies, bound to protein A-agarose beads for 2 h. Immunocomplexes were washed and suspended in 50 mM Tris-HCl (pH 7.5) buffer for the AMPK assay in the presence of 200 μM AMP. Activities were calculated as pmol of phosphate incorporated into the "SAMS" peptide/min/mg of protein subjected to immunoprecipitation as described previously (16). Acetyl-CoA carboxylase was immunoprecipitated from 500 μg of protein with streptavidin and immunoblotted and probed using the ACCα antibody. Acetyl-CoA carboxylase activity was assayed with 100 μg of liver protein and 50 μM of [14C]acetate. Data are expressed as pmol/min/mg.

**Results**

**Socs1-deficient Mice Are Severely Hypoglycemic**—Socs1−/− mice are born normal but rapidly become sick because of inflammation of several organs. Blood analysis revealed that Socs1−/− mice were severely hypoglycemic when they became moribund at 10–14 days of age (Fig. 1A). Typically they displayed blood glucose levels of less than 3 mM. This was compared with 7–8 mM in Socs1+/+ or Socs1+/− littermate controls of the same age.

**Prevention of Inflammation in Socs1−/− Mice Does Not Return Them to Normoglycemia**—Severe hypoglycemia in Socs1−/− mice might be explained by multiorgan inflammation and in particular liver damage. This inflammatory syndrome is dependent on the proinflammatory cytokine IFNγ. Prevention of the inflammatory disease by treating mice with neutralizing antibodies to IFNγ does not return Socs1−/− mice to normoglycemia. Ifng−/− null mice treated with anti-IFN-γ antibodies are incompletely protected from inflammation but survive the neonatal period. They showed evidence of hypoglycemia also (Fig. 2) although much less severe than the mice not treated with anti-IFN-γ antibody. Because antibody-treated Socs1−/− mice eventually go on to develop inflammatory lesions due to the clearance of the antibody (13), we next analyzed Socs1−/− mice that were also deficient for the Ifng gene. These mice are healthy until 1 year of age and then develop low grade inflammation (18). To diminish the effects of genetic heterogeneity Socs1−/− Ifng−/− mice were backcrossed onto the C57BL/6 background for 10 generations. Hypoglycemia was not observed in fed Socs1−/− Ifng−/− mice and was very slight in fasted mice compared with Ifng−/− mice (Fig. 3A).
**Socs1 Deficiency Enhances Hepatic Insulin Signaling**

**Socs1<sup>−/−</sup> and Socs1<sup>−/−</sup> Ifng<sup>−/−</sup> Mice Display Mild Hypoinsulinemia**—A potential cause of hypoglycemia in Socs1<sup>−/−</sup> mice may be increased insulin secretion by SOCS1-deficient β-cells. However both Socs1<sup>−/−</sup> and Socs1<sup>−/−</sup> Ifng<sup>−/−</sup> mice were found to have lower serum insulin levels compared with their respective controls (Figs. 1A and 3B). To test whether hypoinsulinemia was attributed to suppressed insulin secretion, insulin secretory capacity was measured in Socs1<sup>−/−</sup> Ifng<sup>−/−</sup> mice by an intravenous glucose tolerance test and an arginine tolerance test. Interestingly, no significant difference in insulin secretion was seen in the Socs1<sup>−/−</sup> Ifng<sup>−/−</sup> mice (Fig. 3, C and D) suggesting that the mild hypoinsulinemia may be secondary to the mild hypoglycemia and possibly due to enhanced insulin sensitivity.

**Socs1<sup>−/−</sup> Mice Have Suppressed Endogenous Glucose Production—**Ifng deficiency allows Socs1<sup>−/−</sup> mice to grow normally to adulthood and be studied for insulin sensitivity using hyperinsulinemic euglycemic clamps. Socs1<sup>−/−</sup> Ifng<sup>−/−</sup> mice required higher rates of glucose infusion compared with Ifng<sup>−/−</sup> and wild-type mice indicating increased insulin sensitivity. Endogenous glucose production was suppressed in Socs1<sup>−/−</sup> Ifng<sup>−/−</sup> mice, but glucose disposal was not different (Fig. 4). Improved Hepatic Insulin Sensitivity Is Mediated by Elevated IRS-2 Expression and Suppressed Hepatic Gene Expression in Socs1<sup>−/−</sup> Ifng<sup>−/−</sup> Mice—Because of the apparent improvement in hepatic but not peripheral insulin sensitivity we focused on examining mechanisms regulating hepatic insulin sensitivity. Basal (from random fed mice) and insulin-stimulated (following the clamp studies) livers were snap frozen in liquid nitrogen. Hepatic glycogen content was not altered between groups (C57BL/6 306 ± 68 μmol/g/dry mass; Ifng<sup>−/−</sup> 493 ± 74 μmol/g/dry mass; Socs1<sup>−/−</sup> Ifng<sup>−/−</sup> 444 ± 64 μmol/g/dry mass).
Socs1 Deficiency Enhances Hepatic Insulin Signaling

SOCS1 deficiency increases hepatic IRS-2 expression and insulin signaling. Protein extracts from samples taken from mice at 12–15 weeks under basal or clamp conditions and analyzed by Western blot for hepatic IRS-2 expression (A), hepatic insulin-stimulated IRS-2 tyrosine phosphorylation (relative to total IRS-2 expression) (B), and hepatic IRS-2 associated phosphatidylinositol 3-kinase p85 (relative to total p85 expression) (C). D, hepatic insulin-stimulated protein kinase B/Akt phosphorylation; E, hepatic IRS-1 expression. F, representative Western blot of hepatic insulin-stimulated tyrosine phosphorylation, p85, phosphorylated protein kinase B/Akt, relative total IRS-2, p85, and protein kinase B/Akt expression. G, muscle and adipose tissue IRS-1 expression. H, muscle and adipose tissue IRS-2 expression. Values are means ± S.E. (n = 6), *p < 0.05 versus B6 and Ifng−/−.

To investigate the downstream effects of enhanced hepatic insulin sensitivity the mRNA expression of glucose-6-phosphatase and PEPCK were determined. In Socs1 null mice PEPCK expression was significantly reduced during the clamp compared with wild-type and Ifng-deficient mice (Fig. 6) consistent with their reduced glucose production.

**AMPK Is Normal in Socs1 Null Mice**—Recent evidence indicates AMPK may regulate hepatic insulin sensitivity. AMPK is an αβγ heterotrimer consisting of a catalytic subunit (of which there are α1 and α2 isoforms) and two noncatalytic subunits (β, γ) (19). Both metformin (20) (21) and adiponectin (22) increase AMPK activity resulting in improved hepatic insulin sensitivity and reduced hepatic glucose output via the suppression of the gluconeogenic enzymes glucose-6-phosphatase and PEPCK. Hepatic AMPK α1 and α2 activities (pmol/min/mg of protein) were not changed in Socs1−/− mice relative to wild-type and Ifng−/− (AMPK α1, C57BL/6 1.10 ± 0.16; Ifng−/−, 0.78 ± 0.07; Socs1−/− Ifng−/−, 1.26 ± 0.36; AMPK α2, C57BL/6 1.31 ± 0.17; Ifng−/− 0.97 ± 0.19; Socs1−/− Ifng−/−, 1.70 ± 0.33) indicating that the improved insulin sensitivity was not secondary to changes in AMPK.

**SOS3 mRNA Is Strongly Induced in Muscle by Insulin**—Because of the difference in the effect of SOCS1 on insulin sensitivity in liver and muscle, we tested whether SOCS3 expression in muscle might compensate for SOCS1 deficiency and regulate insulin action in muscle. Liver, adipose, and gastro-

Dry mass). Improved insulin sensitivity in Socs1 null mice was associated with elevated IRS-2 protein expression (+39%, p = 0.018 and +67%, p = 0.021, Fig. 5A) and enhanced IRS-2 tyrosine phosphorylation (+68%, p = 0.039 and +75%, p = 0.001, Fig. 5B) relative to wild-type and Ifng−/− mice, respectively. Elevated IRS-2 tyrosine phosphorylation was associated with increased IRS-2 associated phosphatidylinositol 3-kinase p85 (+30%, p = 0.02 and +36%, p = 0.021, Fig. 5C) and phosphorylated protein kinase B/Akt (+43%, p < 0.01 and +41%, p = 0.034, Fig. 5D). SOCS1 deficiency did not alter the expression or phosphorylation of IRS-1 (Fig. 5F). Although the levels of IRS-1 in muscle and adipose tissue were also comparable among the three groups of mice (Fig. 5G), the levels of IRS-2 were increased in these tissues of Socs1−/− Ifng−/− mice (Fig. 5H). Despite increased IRS-2 expression within muscle and adipose tissue insulin stimulated phosphorylation of protein kinase B/Akt was not significantly different (muscle, C57BL/6 1.17 ± 0.84 A.U.; Ifng−/− 1.25 ± 0.22 A.U.; Socs1−/− Ifng−/− 1.30 ± 0.21 A.U.; adipose C57BL/6 1.84 ± 0.57 A.U.; Ifng−/− 1.25 ± 0.16 A.U.; Socs1−/− Ifng−/− 1.18 ± 0.15 A.U.). The data demonstrate that SOCS1 is a critical regulator of IRS-2 but not IRS-1 expression in vivo and suggest improved liver insulin sensitivity in Socs1 null mice is mediated by increased hepatic IRS-2 signaling.
cnenies tissue were removed from mice either at the end of an insulin clamp or without insulin infusion, and SOCS3 mRNA was quantified by reverse transcription PCR. Minor increases in SOCS3 levels were seen in liver and adipose tissue with insulin but much greater increases in SOCS3 expression were observed in muscle (Fig. 7).

**DISCUSSION**

These data show that SOCS1 is a physiological regulator of insulin action. *Socs1*-deficient mice have evidence of increased insulin action resulting in lower blood glucose and insulin levels. Increased rate of glucose infusion required to maintain euglycemia in *Socs1*−/− mice and evidence for greater suppression of hepatic glucose production in response to insulin were observed. Sick *Socs1*−/− mice were hypoglycemic, but healthy mice were much less so. Hypoglycemic mice had suppressed plasma insulin levels consistent with enhanced insulin sensitivity. There was no evidence of increased glucose disposal making it unlikely that insulin action was increased in skeletal muscle or other sites of glucose disposal.

These data are the first detailed metabolic studies in mice genetically deficient in *Socs1*. They are consistent with previous evidence (5–7), particularly inferred from overexpression, that SOCS1 can inhibit insulin signaling. In the liver there was both increased IRS-2 expression and phosphorylation in the setting of a constant infusion of insulin suggesting increased signaling at a proximal step in the insulin signaling pathway. Evidence for indirect effects on insulin sensitivity, for example mediated by adipokines via AMPK, was not found in that AMPK levels and activity were normal. Additionally the rate-determining gluconeogenic enzyme PEPCK was reduced consistent with the observed phenotype of reduced fasting glucose. Expression of gluconeogenic enzymes is reduced by insulin but also by cytokines such as IL-6 (24). Therefore either increased insulin signaling or increased IL-6 signaling or both could be responsible. The role of IL-6 in the regulation of hepatic gluconeogenic enzymes in this setting is unlikely, as basal STAT-3 phosphorylation was undetectable in both wild-type, *Ifng*−/− and *Socs1*−/− *Ifng*−/− animals (data not shown). IL-6 sensitivity may, however, be important in settings in which circulating IL-6 is elevated such as neonatal *Socs1*−/− mice with intact IFNγ. Suppression of endogenous glucose production in clamp studies in response to insulin was also seen. Together these observations are consistent with increased insulin signaling in *Socs1*-deficient hepatocytes. We also sought indirect effects of insulin on endogenous glucose production but found no changes in gluconeogenic fuels such as free fatty acids in *Socs1*−/− *Ifng*−/− mice (data not shown).

It is of interest that deficiency of SOCS1 resulted in liver insulin hypersensitivity, with little effect on peripheral glucose disposal. This was associated with increased tyrosine phosphorylation of IRS-2 (but not IRS-1) and Akt in liver, but no change in intracellular signaling in muscle and adipose tissue as assessed by phosphorylation of Akt. Recent studies have suggested that SOCS1 preferentially binds to the kinase domain of the insulin receptor, which includes the three tyrosine phosphorylation sites (Tyr1146, Tyr1150, and Tyr1151) and that these are critical for the recognition of IRS-2 rather than IRS-1 (7). Furthermore data obtained from genetic ablation of the IRS proteins suggest that IRS-1 is more important in muscle gluconeogenesis, whereas IRS-2 seems to be more prominent in liver glucose metabolism (25–27). Thus, in *Socs1*−/− mice, the preferential increase in IRS-2 phosphorylation is consistent with a preferential effect of SOCS1 on IRS-2 and accounts for the enhanced hepatic insulin sensitivity.

Our data are consistent with recent evidence that reduction in hepatic SOCS1 by intravenous delivery of SOCS1 antisense oligonucleotides can improve insulin action in hyperinsulinemic, severely insulin-resistant *db/db* mice (9). In that study it was suggested that elevated SOCS1 levels may account in part for the insulin resistance of *db/db* mice, but in our study we have found that *Socs1* deficiency increases insulin action physiologically as well as in a pathological state with elevated SOCS1 expression. This is in some ways surprising not only because of the redundancy in the SOCS family but also because constitutive levels of SOCS1 expression in the liver are very low yet apparently sufficient to regulate receptor signaling.

Impaired insulin action in the liver is a critical component of the pathogenesis of type 2 diabetes. Conditional deletion of the *Ir* in liver cells results in severe dysregulation of blood glucose and progressive liver impairment (28), whereas deletion of the *Ir* in muscle results in a much milder phenotype (29). Liver impairment secondary to hepatic insulin resistance is also clinically important and is improved, at least experimentally, by SOCS inhibition (9).

Genetic deficiency of *Irs2* results in many of the features of type 2 diabetes and, unlike *Irs1* deficiency, results in overt diabetes (30). Increased levels of IRS-2 are potentially beneficial not only in liver but also in other tissues including the pancreatic islets, where IRS-2 enhances β-cell function and...
regeneration, and the hypothalamus in which IRS-2 appears to regulate leptin sensitivity (31, 32). We have examined β-cell function in Socs1−/− Ifng−/− mice using glucose-stimulated insulin secretion. Preliminary data in mice on a mixed 129/Sv × C57BL/6 genetic background suggested increased insulin secretion in response to intravenous glucose, consistent with increased β-cell function in the absence of Socs1. However, this was not confirmed in backcrossed C57BL/6 mice, perhaps indicating the effects of background genes. Mice on a mixed genetic background also had more clear cut hypoglycemia than backcrossed mice and increased insulin secretion may have played a part in this. We observed clinically overt hypoglycemia particularly in mice with probable liver failure that is known to be able to cause hypoglycemia. Additionally Socs1−/− mice that are sick have very severe pancreatitis. Histological analysis showed increased numbers of insulin positive cells throughout the parenchyma of the pancreas and increased numbers of insulin-positive ductal cells, consistent with increased β-cell differentiation from precursors (33). This was previously also observed in transgenic ins-IFNγ mice with high local concentrations of IFNγ in the pancreas (23). Therefore it is possible that increased insulin secretion not fully suppressed by hypoglycemia contributes to hypoglycemia in sick Socs1−/− mice as well as insulin sensitivity and impaired hepatic glucose production. In contrast, no abnormality of islet or β-cell number was seen in Socs1−/− Ifng−/− mice.

We also found some evidence for increased expression of SOCS3 in muscle compared with liver, which may also explain the lack of effect of Socs1 deficiency on glucose disposal. From in vitro biochemical studies it is likely that SOCS3 can inhibit binding of both IRS-1 and -2 to the IR and so SOCS3 could potentially substitute for SOCS1. Increased expression of SOCS3 relative to SOCS1 in skeletal muscle could explain the biochemical studies it is likely that SOCS3 can inhibit binding of both IRS-1 and -2 to the IR and so SOCS3 could potentially substitute for SOCS1.

Socs1 deficiency enhances hepatic insulin signaling
