Antisense oligonucleotides against the lipid phosphatase SHIP2 improve muscle insulin sensitivity in a dietary rat model of the metabolic syndrome

Roland Buettner, Iris Ottinger, Christiane Gerhardt-Salbert, Christian E. Wrede, Jürgen Schölmerich, and L. Cornelius Bollheimer

Department of Internal Medicine I, University of Regensburg, Regensburg, Germany

Submitted 31 May 2006; accepted in final form 19 February 2007

Buettner R, Ottinger I, Gerhardt-Salbert C, Wrede CE, Schölmerich J, Bollheimer LC. Antisense oligonucleotides against the lipid phosphatase SHIP2 improve muscle insulin sensitivity in a dietary rat model of the metabolic syndrome. Am J Physiol Endocrinol Metab 292: E1871–E1878, 2007. First published March 27, 2007; doi:10.1152/ajpendo.00263.2006.—The lipid phosphatase SH2 domain-containing lipid phosphatase (SHIP2) has been implicated in the regulation of insulin sensitivity, but its role in the therapy of insulin-resistant states remains to be defined. Here, we examined the effects of an antisense oligonucleotide (AS) therapy directed against SHIP2 on whole body insulin sensitivity and insulin action in liver and muscle tissue in a dietary rodent model of the metabolic syndrome, the high-fat-fed (HF) rat. Whole body insulin sensitivity was examined in vivo by insulin tolerance tests before and after the intraperitoneal application of an AS directed against SHIP2 (HF-SHIP2-AS) or a control AS (HF-Con-AS) in HF rats. Insulin action in liver and muscle was assayed by measuring the activation of protein kinase B (Akt) and insulin receptor substrate (IRS)-1/2 after a portal venous insulin bolus. SHIP2 mRNA and protein content were quantified in these tissues by real-time PCR and immunoblotting, respectively. In HF-SHIP2-AS, whole body glucose disposal after an insulin bolus was markedly elevated compared with HF-Con-AS. In liver, insulin activated Akt similarly in both groups. In muscle, insulin did not clearly activate Akt in HF-Con-AS animals, whereas insulin-induced Akt phosphorylation was sustained in SHIP2-AS-treated rats. IRS-1/2 activation did not differ between the experimental groups. SHIP2 mRNA and protein content were markedly reduced only in muscle. In standard diet-fed controls, SHIP2-AS reduced SHIP2 protein levels in liver and muscle, but it had no significant effect on insulin sensitivity. We conclude that treatment with SHIP2-AS can rapidly improve muscle insulin sensitivity in dietary insulin resistance. The long-term feasibility of such a strategy should be examined further.

Insulin resistance; obesity; high-fat diet; antisense oligonucleotide therapy; SH2 domain-containing lipid phosphatase

OBSERVATIONS AND DISORDERS OF GLUCOSE TOLERANCE are major hallmarks of the so-called metabolic syndrome. This condition can be diagnosed in up to 30% of the elderly in the Western world (12), and it represents a major healthcare challenge because of the difficulty of managing the complex metabolic dysregulations and their associated complications (10).

It is well recognized that both the resistance of insulin-dependent tissues to the metabolic actions of insulin and an impairment of the pancreatic islets’ insulin secretory capacity contribute to the development of glucose intolerance and eventually type 2 diabetes mellitus seen in many patients with the metabolic syndrome. The molecular basis of insulin resistance has been examined closely in the last 15 years, and it is clear now that visceral obesity and elevated free fatty acids lead to perturbations of the intracellular insulin signaling cascade. Impairments of the insulin receptor-associated tyrosine kinase activity, the insulin-induced phosphatidylinositol (PI)-3-kinase, and Akt activation and of consecutive downstream metabolic pathways have been described (recently reviewed in Ref. 9).

Phosphatidylinositol 3,4,5-phosphate (PIP3), the product of the PI 3-kinase reaction, plays a major role as second messenger in the insulin signaling pathways. Recently, an SH2 domain-containing lipid phosphatase (SHIP2) catalyzing the dephosphorylation of PIP3 in insulin-sensitive tissues as liver and muscle has been characterized (7, 19). Metabolic actions of insulin are impaired in myocytes and adipocytes overexpressing SHIP2 (20, 26), and the transgenic expression of SHIP2 in rodent livers impairs hepatic insulin sensitivity (11). SHIP2 gene knockout mice show normal glucose and insulin tolerance, but they are resistant to diet-induced obesity (22).

Clinical studies have examined whether SHIP2 might be a candidate gene for human disorders of glucose tolerance. A mutation in the 3′-untranslated region of the SHIP2 gene was detected in a Caucasian with type 2 diabetes (15), and recent findings in a Japanese population indicate that the L632I polymorphism of the SHIP2 gene might be associated with a lower prevalence of type 2 diabetes (14). These findings implicate a role for SHIP2 in the development of human insulin resistance.

Considering these results, the modulation of SHIP2 activity might represent a new therapeutic approach for insulin-resistant states (2). The proof of principle has been provided by Bertelli and coworkers (3), who were able to improve insulin sensitivity in a muscle denervation model by using antisense oligonucleotides (AS) to block SHIP2 protein synthesis. To our knowledge, such an antisense strategy has not been examined in diet-induced insulin resistance yet.

High-fat feeding leads to an obese and insulin-resistant phenotype that closely resembles the human metabolic syndrome (23); the animals also show similar disorders of insulin signaling (25, 27, 28). In this study, we have therefore investigated the effect of SHIP2-AS on insulin action and SHIP2 mRNA expression in high-fat-fed rats.

MATERIALS AND METHODS

Experimental animals. Six-week-old male Wistar rats were purchased from Charles River (Sulzfeld, Germany). Rats were caged with free access to water and subjected to different dietary regimes as

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
described below. Diets were prepared in pellet form by Altromin (Lage, Germany). Animals were held on a 12:12-h light-dark cycle. All animal procedures were approved by the local animal rights committee and complied with the German Law on Animal Protection and the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals, 1996.

Experimental design. After 3 days of acclimatization, the rats were fed ad libitum with either the standard rodent chow (SC, fat content 11 energy percent, based on lard, n = 12) or a high-fat diet (HF, fat content 43 energy percent, based on lard, n = 12). After 12 wk, tail blood was drawn from all animals for the analysis of basal serum parameters, and an insulin tolerance test (see below) was performed. On the evening of the next day, HF and SC animals were injected intraperitoneally with 200 μl of dilution buffer (10 mmol/l Tris-HCl, 1 mmol/l EDTA, pH 7.4) containing 4 nmol SHIP2-AS (see below, n = 6) or the same amount of the control oligonucleotide (see below, n = 6). The animals were fasted overnight, and the antisense/control oligonucleotides were injected a second time on the next morning. After 2 h, an insulin tolerance test was performed. Later (2 wk), the animals were injected with the antisense/control oligonucleotides as described above; they were then killed by CO₂ gassing. Liver and muscle tissue samples were clamp-frozen in penetrating χRNA purification or lysis for protein assays. Other animals treated in the same manner were used for organ-specific assays of insulin action as described below.

Insulin tolerance test. Fasting glucose levels were measured repeatedly for at least 30 min with a hand-held glucometer (AccuTrend; Roche Diagnostics, Mannheim, Germany) from whole blood drawn from the tail tip capillary region. After a stable baseline glucose level was established, the animals were injected intraperitoneally with 0.15 U/kg body weight insulin (Aventis, Frankfurt, Germany). Whole blood glucose levels were then monitored every 10 min for 30 min. The total glucose disposal induced by insulin was calculated from the area under the glucose/time curves.

Organ-specific in vivo insulin action assays and immunoblotting. In vivo insulin action was assayed by measuring the insulin-induced Akt and insulin receptor substrate (IRS)-1/2 activation based on the method described by Anai et al. (1). After an overnight fast, rats were anesthetized with pentobarbital sodium (50 mg/kg body wt). The abdomen was opened, and a Teflon catheter was inserted in the portal vein. Saline (4 ml of 0.9% NaCl) with or without 10⁻⁶ mol/l of human insulin (Aventis, Frankfurt, Germany). Whole blood glucose levels were then monitored every 10 min for 30 min. The total glucose disposal induced by insulin was calculated from the area under the glucose/time curves.

Table 1. Basal parameters of rats fed with standard chow or a high-fat diet

<table>
<thead>
<tr>
<th></th>
<th>Standard Chow</th>
<th>High-fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final wt, g</td>
<td>512±27</td>
<td>597±64*</td>
</tr>
<tr>
<td>Plasma glucose, mmol/l</td>
<td>4.8±0.2</td>
<td>5.3±0.8</td>
</tr>
<tr>
<td>Plasma insulin, pmol/l</td>
<td>490±160</td>
<td>720±230</td>
</tr>
<tr>
<td>HOMA index</td>
<td>16±26</td>
<td>24±3*</td>
</tr>
<tr>
<td>Glucose disposal, %</td>
<td>−13.8±7.3</td>
<td>0.7±7.7*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 rats in each group. HOMA, homeostasis model assessment. *P < 0.05 compared with standard chow.
Serum parameters. Plasma glucose was analyzed using a standard hexokinase assay (Roche Diagnostics). Plasma insulin was measured using a rat-specific ELISA kit (Mercodia, Uppsala, Sweden). The homeostasis model assessment (HOMA) index was calculated as follows: HOMA index = glucose (mmol/l) × insulin (pmol/l)/155 (16).

mRNA analysis. Total RNA from liver and muscle specimens was isolated using a Qiagen RNeasy kit (Qiagen, Hilden, Germany). Real-time RT-PCR was performed as described in detail elsewhere (4). In brief, first-strand cDNA was synthesized from equal amounts of total RNA by priming with arbitrary hexamers. For subsequent PCR amplification (Standard RT-PCR and LightCycler system; Roche Diagnostics), the following primer pairs (1 μmol/l) were employed: 1) SHIP2, 5′-TTG AAC CCC AGG TTG AAG TC-3′ (sense) and 5′-GCC ACT TTG TTC TTG GTG GT-3′ (antisense); 2) 18S rRNA, 5′-TCA AGA ACG AAA GTC GGA G-3′ (sense) and 5′-GGA CAT CTA AGG GCA TCA CA-3′ (antisense). After verification of the RT-PCR products by gel electrophoresis, a LightCycler analysis was performed with the same temperature protocol. The formation of primer-dimers was ruled out by melting-curve analysis. The cDNA content for a specific gene in each sample was semiquantitatively assessed by comparing the experimentally determined crossing point with the crossing points and respective concentrations of a pooled standard cDNA as described previously (4). All results were normalized by the 18S rRNA content to ensure comparability.

Statistical methods. To obtain representative data, all experiments were performed with six animals per study group unless otherwise stated. Data are presented as means ± SD or as 95% confidence intervals. Statistically significant differences were analyzed using an exact Fisher-Pitman-Permutation Test. The significance level was set to P < 0.05.

RESULTS

HF phenotype. After 12 wk of ad libitum HF, the animals weighed 15–20% more than age-matched control rats fed with SC (P < 0.05). Fasting glucose and insulin levels were slightly, but not significantly, elevated. The HOMA index was ~55% higher in HF animals (P < 0.05). These results indicated whole body insulin resistance in HF animals, which was confirmed by insulin tolerance tests. Here, the insulin-induced glucose disposal was significantly lower in HF rats (Table 1).

Effect of SHIP2-AS on whole body insulin sensitivity. SC and HF rats were treated with the SHIP2-AS (SC-SHIP2-AS and HF-SHIP2-AS, respectively) or the control oligonucleotide (SC-Con-AS and HF-Con-AS, respectively) as described above. After two injections, blood samples were drawn, and insulin tolerance tests were performed. Neither the fasting glucose levels (SC-SHIP2-AS: 4.7 ± 0.3 mmol/l, HF-SHIP2-AS: 4.6 ± 0.3 mmol/l, P = not significant) nor the fasting insulin levels (SC-SHIP2-AS: 500 ± 140 pmol/l, HF-SHIP2-AS: 620 ± 150 pmol/l, P = 0.09) had changed significantly in the SHIP2-AS-treated rat groups, although there was a slight trend downward. After the insulin challenge, SC-SHIP2-AS rats did not show an increased insulin sensitivity (Fig. 1A). In HF-SHIP2-AS, blood glucose levels fell more rapidly in the insulin tolerance tests and were significantly lower after 30 min compared with HF-Con-AS (−19 ± 6%, P < 0.05; Fig. 1B), and the total insulin-induced glucose disposal estimated from these tests was significantly enhanced to −6.6 ± 2.7%. These results indicate that dynamic insulin action was improved in

![Fig. 2. Insulin-induced activation of protein kinase B (Akt) in HF-fed rats treated with SHIP2-AS. Insulin-induced Akt activation was assayed in liver (A) and muscle (B) tissue before and after an insulin bolus (filled bars) or a saline bolus (open bars) given in the portal vein in vivo. Values are means ± SD of the Ser473-phospho-Akt/total Akt-ratio as derived from the densitometric analysis of liver homogenate Western blots; n = 6 in all groups. P < 0.05, basal vs. insulin-stimulated values (¶) and HF-SHIP2-AS vs. HF-Con-AS (*).](http://ajpendo.physiology.org/doi/abs/10.1152/ajpendo.00702.2006)
HF-SHIP2-AS rats, although the basal insulin and glucose values had not improved significantly.

**Effect of SHIP2-AS on liver and muscle insulin action.** To verify the insulin-sensitizing effect of SHIP2-AS, in vivo insulin action assays were performed in liver and muscle tissue samples of HF animals (Fig. 2). In liver, insulin stimulated the phosphorylation of Akt similarly in HF-Con-AS and HF-SHIP-AS rats (2.2 ± 0.3- and 2.9 ± 0.4-fold, respectively). Contrasting this, in muscle tissue, only HF-SHIP-AS animals showed a clear induction of Akt phosphorylation by insulin stimulation (4.3 ± 1.3- vs. 1.6 ± 0.8-fold in HF-Con-AS, P < 0.05). When looking at standard-fed animals (Fig. 3), insulin-induced Akt activation in liver in SC-Con-AS was comparable to that observed in HF-Con-AS (2.6 ± 0.3-fold), whereas Akt activation in muscle (4.8 ± 0.6-fold vs. saline control) was clearly higher than in the HF control group. Treatment with SHIP2-AS did not lead to significant changes in insulin-induced Akt activation in neither tissue of SC animals.

To rule out an influence of SHIP2-AS on insulin signaling upstream of Akt, we also analyzed insulin-induced IRS-1 and IRS-2 activation in liver and muscle tissue of HF rats (Fig. 4). In liver, HF-SHIP2-AS and HF-Con animals showed a 2.3- and 1.6-fold increase in IRS-1/2 tyrosine phosphorylation after insulin stimulation; the slight difference between the two groups was not statistically significant. In muscle, we observed a similar induction of IRS-1/2 phosphorylation in both HF-SHIP2 and HF-Con rats of about fivefold.

**Effect of SHIP2-AS on SHIP2 mRNA expression.** SHIP2 mRNA expression was measured in liver and muscle from HF and SC animals treated with SHIP2-AS and Con-AS as described above by quantitative Lightcycler RT-PCR (Fig. 5). Although there was a slight trend toward lower SHIP2 mRNA levels in the livers of HF-fed animals compared with SC, this effect was not found to be statistically significant. In HF rats, SHIP2-AS induced a reduction of SHIP2 mRNA expression by −65 ± 12% in muscle (P < 0.05 compared with Con-AS). In liver, the reduction compared with HF-Con-AS was less distinct (−38 ± 15%), but it remained statistically significant (P < 0.05).

Similarly, in SC rats, SHIP2-AS reduced SHIP2 mRNA abundance by 55 ± 12% in liver and 49 ± 8% in muscle compared with SC-Con-AS (P < 0.05).

**Effect of SHIP2-AS on SHIP2 protein expression.** To elucidate whether the transcriptional effects of SHIP2-AS also translated to SHIP2 protein levels, we quantified SHIP2 protein content by immunoblotting. As to be expected from the mRNA data, there was no significant change of SHIP2 abundance induced by HF feeding. In livers from HF animals, SHIP2-AS slightly, but significantly, reduced SHIP2 levels (Fig. 6), whereas in SC rats SHIP2-AS treatment led to an ~30% reduction of hepatic SHIP2 protein content (P < 0.05). As Fig. 6 shows, SHIP2 protein levels were reduced ~0.6-fold in muscle tissue taken from HF-SHIP2-AS compared with HF-Con-AS (P < 0.05); this similar effect was observed in SC animals.

**DISCUSSION**

In this study, we examine the effects of an oligonucleotide-based antisense strategy targeted against the lipid phosphatase...
SHIP2 in a HF diet rat model of the metabolic syndrome. We found evidence for a rapid improvement of diet-induced insulin resistance by this intervention. This became apparent by 1) an increased insulin-induced glucose disposal in insulin tolerance tests and 2) an elevated insulin-induced phosphorylation of Akt in muscle tissue of animals treated with the SHIP2-AS oligonucleotide two times within 12 h. IRS-1/2 tyrosine phosphorylation in muscle was not altered by SHIP2-AS, which is consistent with a specific antisense effect, since SHIP2 inhibition should lead to increased PIP3-levels, thereby activating insulin signaling downstream of the IRS molecules. Interestingly, the improvements in these dynamic measures of insulin action could not be clearly anticipated from the basal glucose and insulin levels measured in SHIP2-AS-treated animals, although the trendwise reduction of fasting serum insulin might be interpreted as a sign in this direction.

The study is limited by certain methodological constraints. First, because we did not perform euglycemic hyperinsulimemic clamp experiments, we cannot rule out hypoglycemia-induced counterregulatory stress hormone responses that might have influenced the glucose profiles in the insulin tolerance tests. However, because such reactions can be expected to be related to the degree of hypoglycemia, they should mainly blunt putative insulin-sensitizing effects. Hence, although such clamp experiments certainly would be necessary for an exact characterization of whole body insulin sensitivity in the experiments, we do not think they would change the conclusion that SHIP2-AS treatment enhances overall insulin-induced glucose disposal. Second, SHIP2 activity levels were not directly measured in the insulin-dependent tissues. This method is not well established, and, in our opinion, the data presented can be sufficiently interpreted without these results. Protein and expression analysis showed a clear reduction of SHIP2 protein and mRNA abundance in muscle, and, together with the increase in insulin-stimulated Akt phosphorylation, this is consistent with a functionally relevant decrease of SHIP2 activity in this tissue.

The data obtained on liver tissue are more difficult to interpret. There was only a moderate reduction of hepatic SHIP2 mRNA expression, which correlated with a modest improvement of insulin action when comparing Akt activation...
in HF-SHIP2-AS and HF-Con-AS animals. Because this effect was much less pronounced than the SHIP2-AS-induced increase of muscular Akt activity, it might seem appropriate to conclude that the effect on whole body glucose disposal we observed was mainly the result of the SHIP2-AS effects on muscle. However, due to the fact that HF feeding in itself did not clearly reduce hepatic insulin-induced Akt-activation, our data do not allow clear conclusions about SHIP2-AS effects in liver. We cannot refute the notion that other hepatic components of the insulin signaling system might have been modified. The observation that the hepatic insulin resistance usually associated with HF feeding when using whole body procedures in vivo (e.g., see Ref. 17) was not detected when looking at direct hepatic effects of insulin administered intraportally is actually not new (1, 6) and hints at the ongoing discussion about direct and indirect effects of insulin on the liver. These issues may at least partially be explained by central mechanisms or circulating factors that interfere with insulin signaling and/or hepatic glucose production in the whole body setting (5, 18). We cannot rule out, however, that the insulin dose we used might have sufficed to maximally stimulate hepatic insulin signaling, thereby obscuring mild insulin resistance.

Our results match well with data from the first study examining a SHIP2 antisense therapy by Bertelli et al. (3). These authors have shown that SHIP2-AS treatment can restore the levels of phosphorylated phosphatidylinositol5,4,5-trisphosphate at least partially and improve glucose uptake in another model of insulin resistance, the denervated rat muscle (3). Hepatic or muscle Akt phosphorylation was not measured in this study, limiting comparability with respect to direct cellular insulin actions. To our knowledge, apart from the study cited above, no other paper has so far been published examining an SHIP2 antisense strategy for the treatment of insulin resistance in vivo. Several authors have examined the effect of dominant-negative SHIP2 mutants on insulin sensitivity in different cell culture types and in vivo. In fibroblasts, L6 myotubes and 3T3-L1 adipocytes, this approach has demonstrated an increase in insulin-induced Akt phosphorylation, arguing for an enhancement of insulin action by endogenous SHIP2 downregulation (20, 21, 26). Some controversy exists, however, about the role of SHIP2 in 3T3-L1 cells, since other researchers did not observe relevant changes of insulin signaling in this cell line after silencing of the SHIP2 gene by RNA interference (24).

The downregulation of hepatic SHIP2 in db/db mice by adenoviral transfer of a dominant-negative mutant led to an improvement of both oral glucose tolerance and insulin-induced Akt phosphorylation in liver (11). As to be expected from the adenoviral gene transfer method, insulin signaling in muscle and adipocytes was not directly influenced in this model. Taking this into account, it is interesting to note again that, in our study, the main effects of SHIP2-AS treatment were observed on insulin action in skeletal muscle and not in liver. It can be speculated that an increase of insulin sensitivity induced by some form of SHIP2 antagonism in either organ can improve systemic insulin resistance.

Two different SHIP2 knockout mice strains have been generated. Clement et al. (7) described fatal hypoglycemias resulting from severe insulin sensitivity in their original model, but the interpretation of these results is made difficult by the fact that the Phox2a gene was also unintentionally deleted in these animals (8). Recently, Sleeman et al. (22) have questioned whether SHIP2 has a dominant role in modulating glucose homeostasis due to results from a new knockout mouse in which only the SHIP2 gene was ablated because SHIP2 null mice fed with standard rodent diet did not show overt hypo-
glycemia. Akt phosphorylation in muscle and liver was increased in these animals, hinting at an increased insulin sensitivity that did not become functionally relevant. When placed on a HF diet, SHIP2 null mice gained less weight and did not develop elevated serum lipids, insulin, or glucose levels. Supporting our results, wild-type mice fed with a HF diet showed a more pronounced decrease of insulin action as assayed by Akt activation in skeletal muscle compared with liver tissue, and this HF-diet-induced insulin resistance in muscle was ameliorated in SHIP2 knockout animals. These previous results are consistent with our observation that treatment of SC-fed rats with SHIP2-AS did not relevantly change the insulin-induced glucose disposal. Taken together, these data are consistent with the interpretation that a decrease in SHIP2 activity will become significant only in insulin-resistant states.

The transient AS treatment was tolerated without obvious side effects in our study, but, given the ubiquitous expression of SHIP2, the question whether this strategy is tolerable in the long term becomes important. To date, no studies are available to answer this specific question. Clinical studies have shown that antisense strategies can generally be used for the treatment of human disease without major side effects (reviewed in Ref. 13), but further research will have to demonstrate the feasibility of a prolonged SHIP2-AS application for the in vivo therapy of insulin-resistant states.

In conclusion, this study presents data arguing for an important role of SHIP2 in diet-induced insulin resistance. As far as can be deduced from insulin tolerance assays, an AS therapy directed against SHIP2 can ameliorate the decrease in insulin-induced glucose disposal observed after HF feeding; this effect can be retracted on a molecular level by an improvement in insulin-induced Akt phosphorylation. These findings apply mainly to skeletal muscle in this model, whereas SHIP2-AS effects on the liver were not conclusive. Further studies are needed to characterize these thoroughly and to assess the long-term safety and efficacy of such a new putative therapeutic strategy.

ACKNOWLEDGMENTS

We are indebted to Dr. Christa Buechler for reading and discussing the manuscript.

GRANTS

This work was supported by a grant from the Else Kröner-Fresenius-Stiftung, Bad Homburg, Germany.

REFERENCES


