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Short Communication

Effects of insulin-sensitising agents in mice with hepatic insulin resistance

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Abstract

Aims/hypothesis. The metabolic abnormalities of insulin resistance are ameliorated by insulin sensitisers via different mechanisms. Metformin decreases hepatic glucose output, whereas rosiglitazone (RSG) is an agonist for peroxisome proliferator activated receptor (PPAR)γ, highly expressed in fat. To gain insight into the mechanisms of action of these drugs, we compared their actions in two models of insulin resistance: the obese, hyperglycaemic *ob/ob* mouse and the liver specific insulin receptor knockout (LIRKO) mouse.
Methods. Control, *ob/ob*, and LIRKO mice were divided into three groups that received metformin (300 mg/kg body weight/day), RSG (3 mg/kg body weight/day), or placebo for 3 weeks.
Results. In the presence of the severe hepatic insulin resistance of the LIRKO mouse, neither metformin nor RSG had any significant effect on glucose or insulin tolerance tests. On the other hand, RSG decreased serum concentrations of total cholesterol, LDL, and HDL in LIRKO mice. Adipocyte PPARγ gene and protein expression, and adipocyte size were all increased in LIRKO mice treated with RSG, whereas fat-cell size in control animals was decreased by RSG.
Conclusion/interpretation. TZDs probably improve some lipid parameters of the dysmetabolic syndrome associated with diabetes mellitus even in the presence of absolute hepatic insulin resistance, but both metformin and TZDs require an operating insulin signalling system in the liver for their effects in glucose homeostasis. [Diabetologia (2004) 47:407–411]

Keywords Thiazolidinedione · Rosiglitazone · Metformin · Diabetes mellitus

The metabolic abnormalities of insulin resistant states are ameliorated by insulin-sensitising agents, such as metformin and the thiazolidinedione (TZD) rosiglitazone (RSG) through different mechanisms [1]. Metformin has been shown to act primarily by decreasing hepatic glucose output, whereas TZD treatment increases peripheral glucose disposal [2]. This effect of TZDs is mediated by binding to the peroxisome proliferator activated receptor (PPAR)γ, which is most abundant in fat [3]; the mechanism of metformin action remains uncertain, but presumably involves the liver [4].

To gain insight into the physiologic and molecular mechanism of action of these drugs, we have used a new mouse model of isolated severe hepatic insulin resistance, the liver specific insulin receptor knockout (LIRKO) mouse [5]. The LIRKO model was chosen because it has a liver specific form of insulin resistance such that any effects produced by these compounds must be attributed to a mechanism that does not involve direct insulin signalling in the liver. To es-

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Abbreviations: TZD, Thiazolidinedione · RSG, rosiglitazone · LIRKO, liver specific insulin receptor knockout

tablish the effects of insulin-sensitising agents on the physiologic parameters of insulin resistance, we evaluated serum concentrations of insulin, glucose, lipids, and NEFA, and did an insulin tolerance test (ITT) and glucose tolerance test (GTT).

Materials and methods

Animals and treatment. The LIRKO mouse was generated using the Cre/loxP system for site-specific excisional DNA recombination by crossing mice carrying a floxed insulin receptor, IR (*lox/lox*) with mice carrying the Cre transgene driven by the albumin promoterand heterozygous for the floxed allele IR (*lox*/+) [6]. *Ob/ob* mice were on a C57bl/6 background purchased from Jackson Laboratory, Bar Harbor, Maine.

Animals were housed in virus-free facilities on a 12-h light to dark cycle (0700 on, 1900 off) and were fed a standard rodent chow (Mouse Diet 9F, PMI Nutrition International: percent of calories from carbohydrates, 56.5%; fat, 21.6%; and protein, 21.9%) ad libitum. All protocols for animal use and killing were approved by the Animal Care Committee of the Joslin Diabetes Center and were in accordance with NIH guidelines. Genotyping was done by PCR using genomic DNA isolated from the tail tip [6].

Drug treatment. Two-month old male LIRKO (n=48), control (Ctrl) (n=37) and *ob/ob* (n=9) mice were treated for 3 weeks with either RSG (3 mg/kg body weight) or metformin (300 mg/kg body weight). The drugs were thoroughly mixed with powdered chow and distributed into individual feeding chambers, with the amounts of food weighed to insure equal drug distribution to each mouse. Food left by the mice was weighed biweekly, and no difference was found between either the control or LIRKO mice, or those treated with either drug.

Analytical procedures. Blood glucose was measured from whole venous blood using an automatic glucose monitor (Elite, Bayer, New Haven, Conn., USA). Insulin concentrations in serum were measured by ELISA using mouse insulin as a standard (Crystal Chem, Downers Grove, Ill., USA). Triglyceride concentrations in serum from fasted animals were measured by colorimetric enzyme assay using the GPO-Trinder Assay (Sigma Chemical, St. Louis, Mo., USA). Non-esterified fatty acid concentrations were analysed in serum from fasted animals using the NEFA-Kit-U (Wako, Richmond, Va., USA). The Beckman CX7 analyser (Pasadena, Calif., USA) was used to measure liver function tests and the lipid profile. Glucose tolerance tests (GTT) were done on mice that had been fasted overnight for 16 h, whereas insulin tolerance tests (ITT) were done on mice in a fed state at 2:00 pm. Animals were injected with either 2 g/kg body weight of glucose or 1.5 U/kg body weight of human regular insulin (Lilly) in the intraperitoneal cavity for the GTT and ITT, respectively. Glucose concentrations were measured from blood collected from the tail immediately before and at 15-, 30-, and 60-min intervals after the injection; and for the ITT, additionally at 120 min.

Immunoprecipitation and western blot analysis. Chemicals were obtained from Sigma Chemical (St. Louis, Mo., USA), unless otherwise noted. Tissues were removed and homogenised using a Polytron in homogenisation buffer (50 mmol/l Tris [pH 7.8], 2% SDS, 10% glycerol, 10 mmol/l sodium pyrophosphate, 0.1 mol/l sodium fluoride, 10 mmol/l EDTA, 6 mol/l urea, 10 mmol/l sodium orthovanadate, 10 µg/ml leupeptin, and 2 mmol/l PMSF). Samples were solubilised for 30 min on ice, and particulate matter was removed by centrifugation at 4°C. Western blot analysis was done on at least duplicate individuals of each genotype, and quantified using scanning densitometry, and ImageQuant version 4.0 software.

Histopathology. The liver and epididymal fat pads were isolated immediately after killing the mice, then dried, and weighed. Tissues were fixed in 10% phosphate buffered formalin (Fisher Scientific, Pittsburgh, Pa., USA) and embedded in paraffin. Staining of the sections with haematoxylin/eosin and periodic acid-Schiff (PAS) reagent was done using standard techniques. Immunohistochemistry was done using cleaved anti-caspase-3 at a 1:500 dilution (Cell Signalling Technology, Beverly, Mass., USA) and the AEC Kit (Dako Envision System, Toronto, Ontario).

Statistical analysis. Statistical analysis was done with SigmaPlot 2000 for Windows Version 6.00 (SPSS, Chicago, Ill., USA) using a two-tailed unpaired Student's t test. A probability value of less than 0.05 was considered significant.

Results

The physiologic effects of the disruption of insulin signalling in liver of the LIRKO mouse have been well characterised and include dramatic insulin resistance, severe glucose intolerance, and a failure of insulin to suppress hepatic glucose production and regulate hepatic gene expression [5]. The GTT done on 2-month-old male LIRKO mice showed mild fasting hyperglycaemia and pronounced glucose intolerance throughout the 2-h time course of the assay (Fig. 1). Treatment of the LIRKO mice with either RSG or metformin for 3 weeks had no effect on the GTT (Fig. 1a, b), ITT, or the high serum glucose and insulin concentrations.

To ascertain that the mice were indeed ingesting the insulin-sensitising drugs at an effective dose, we used *ob/ob* mice which lack a functional leptin gene and are morbidly obese, severely insulin resistant, and hyperglycaemic [7]. Treatment with either metformin or RSG decreased the blood glucose concentrations of the *ob/ob* mice from 19.1±1.1 mmol/l to 7.8±0.2 mmol/l, a concentration equivalent to that of the control mice (Fig. 1c). Thus, under conditions where *ob/ob* mice show a major improvement of hyperglycaemia by either metformin or RSG, both drugs fail to affect glucose metabolism in the LIRKO mouse.

Lipid profile with RSG treatment. Insulin-resistant states are associated with a dysmetabolic syndrome that includes alterations in lipid metabolism. LIRKO mice had a threefold increase in low-density lipoprotein (LDL) cholesterol compared with the control mice, with normal total cholesterol (TC) and high-density lipoprotein (HDL) cholesterol. The increased LDL cholesterol was decreased significantly by RSG.

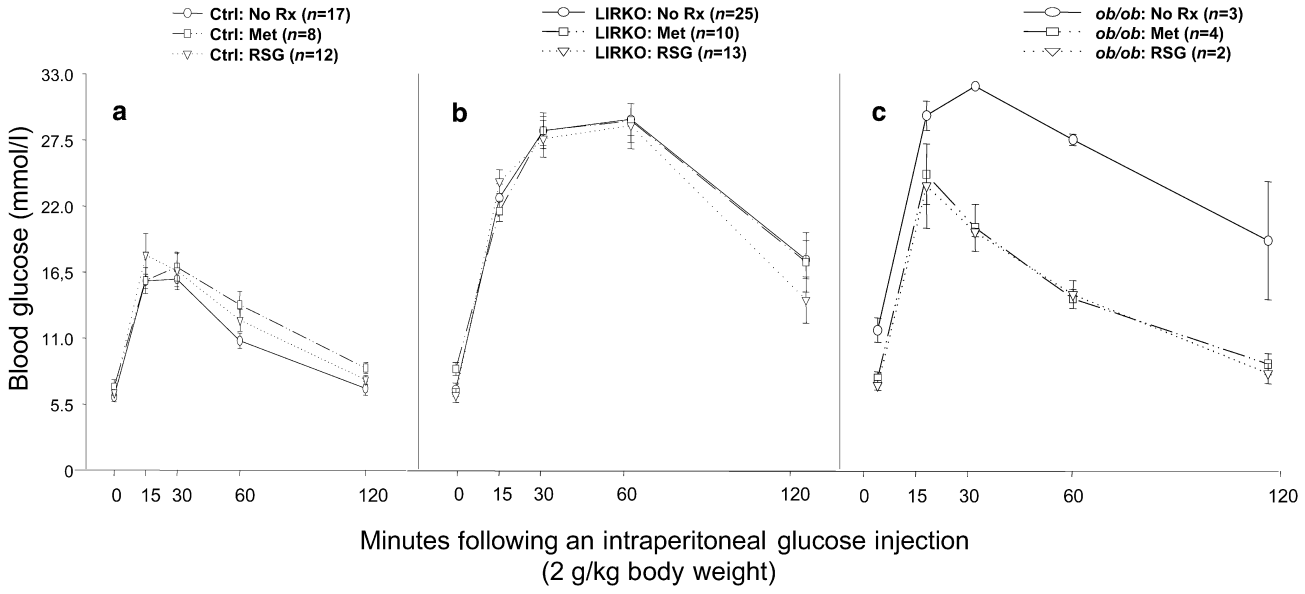

Fig. 1a–c. Glucose tolerance tests show insulin resistance of *ob/ob*, but not LIRKO, mice improved with either metformin (met) or rosiglitazone (RSG) treatment (Rx). Glucose tolerance tests were done on 2-month-old male (**a**) $IR^{(lox/lox)}$ (Ctrl), (**b**) LIRKO, and (**c**) *ob/ob* mice that had been fasted for 16 h, after being treated for 3 weeks with either placebo (*white diamonds*), metformin (*white squares*) (300 mg/kg body weight), or RSG (*white triangle*) (3 mg/kg body weight). Animals were injected intraperitoneally with 2 g/kg body weight of glucose. Blood glucose was measured immediately before injection and 15, 30, 60, and 120 min after the injection. Results are expressed as mean blood glucose concentration ± SEM

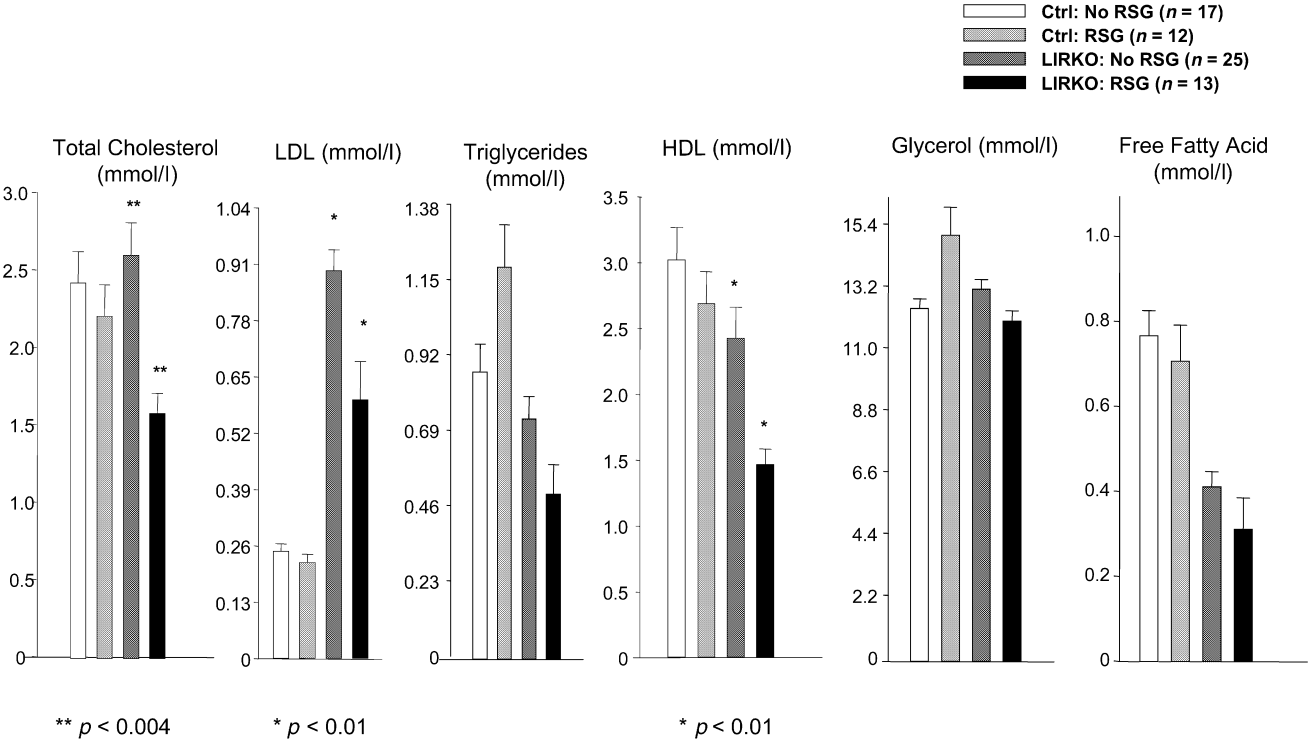

Fig. 2. Fasted lipoprotein profile shows a decrease in total cholesterol, LDL cholesterol, and HDL cholesterol in LIRKO mice treated with rosiglitazone (RSG). Lipoprotein profile was determined in 2-month-old male $IR^{(lox/lox)}$ (Ctrl) and LIRKO mice that had been fasted for 16 h, after being treated for 3 weeks with either placebo, metformin (300 mg/kg body weight), or RSG (3 mg/kg body weight). Control mice, placebo-treated (*white bars*); control mice, RSG-treated (*light gray bars*); LIRKO mice, placebo-treated (*dark gray bars*); LIRKO mice, RSG-treated (*black bars*)

Likewise in both LIRKO mice and control mice, both TC and HDL cholesterol decreased with RSG by approximately 30%, whereas the HDL/TC ratio was unchanged. Fasting serum triglycerides and NEFA in untreated LIRKO mice were approximately 35% lower than in the control mice and were unchanged by RSG treatment in either the LIRKO or control mice (Fig. 2).

Effect of RSG treatment. There was no significant difference in body weight between LIRKO and control mice at 2 months of age. Neither the fasting nor fed body weights of LIRKO or control mice changed during 3 weeks of treatment with RSG or metformin. However, RSG decreased the weight of epididymal fat pads in the control mice 22% ($p<0.003$), while increasing fat-pad weight in the LIRKOs by 32% ($p<0.04$). RSG treatment also decreased average fat-cell size in control, but increased fat-cell size in the LIRKO mice, as estimated by quantitative analysis of haematoxylin and eosin-stained fat pads.

Immunohistochemistry staining of fat pads from control mice with the apoptosis marker cleaved caspase-3 showed evidence of increased cell death after RSG treatment. Western blot analysis of fat-pad protein from both control and LIRKO mice showed that death receptor Fas and its ligand FasL were increased by RSG treatment. The precursor form of caspase-3 was also strongly increased by RSG in the control mice and increased to a lesser extent in the LIRKO mice. Similar studies of the liver failed to show any changes. These data suggest that RSG treatment can induce increased rates of apoptosis in fat but not liver cells, and that this can contribute to the ability of the drug to change cell populations in a relatively short period of time.

Discussion

We have shown that lipid abnormalities, but not impaired glucose homeostasis associated with hepatic insulin resistance, are improved by RSG. Additionally, the high serum glucose and insulin concentrations in both the fasted and fed states in LIRKO mice are refractory to treatment with either metformin or RSG. This dichotomy between the drug effects on lipid metabolism and glucose metabolism indicates that intact insulin receptor function in liver is critical to the effects of RSG and metformin on glucose homeostasis, but not necessarily for the mechanism of action of RSG's effect on lipid metabolism.

The fact that the effect of RSG on the adipocyte is intact in the LIRKO mouse indicates that the normal cross talk between the adipocyte and liver in the insulin sensitising effect of RSG is interrupted by knockout of IR in liver. Further proof that the IR is essential to the insulin sensitising effect of RSG comes from a recently published article describing two patients with mutations in the IR gene in the tyrosine kinase domain [8]. In these patients with extreme insulin resistance, RSG produced no change in oral or intravenous GTT. Unlike our study, however, in these patients there was also no change in TG, or HDL or LDL cholesterol suggesting that if the IR is intact in organs other than the liver, as in the LIRKO model, this is sufficient for RSG to have an effect on lipid metabolism. The presence of some adipose tissue also seems to be necessary for the antidiabetic, but not the hypolipidemic, effects of RSG, as shown by the lack of effects of these drugs in A-ZIP/F-1 mice that lack adipose tissue [9]. Overall, these studies indicate that both intact adipose tissue and liver are necessary for the antidiabetic action of RSG, however only an intact liver is necessary for the lipid lowering effects of RSG.

The effect of RSG on lipid metabolism was not associated with a change in body weight. Epididymal fat-pad weight, on the other hand, was decreased by treatment with RSG in the control mice, while it was increased in the LIRKO mice. This was due primarily to a change in adipocyte cell size that followed the same pattern as the epididymal fat-pad weight with RSG treatment in both the control and LIRKO mice.

One mechanism contributing to the decrease in the epididymal fat pad weight and adipocyte cell size by RSG treatment in the control mice could be increased apoptosis, as suggested by immunohistochemistry staining with cleaved caspase-3 and western blot analysis with the precursor form of caspase-3, Fas, and Fas ligand. These three apoptosis markers were also increased by RSG treatment in epididymal fat from LIRKO mice, although to a lesser extent; however, in the RSG treated LIRKO mice this was associated with an increase in epididymal fat-pad weight and adipocyte cell size. These different responses to RSG could reflect the differences in circulating insulin concentrations, which are very high in LIRKO mice and normal in control mice. Thus, this effect of RSG to increase adipocyte weight and size, despite a mild increase in apoptosis would only be expected to occur in models that have high insulin concentrations; in other situations one would expect that no increase in apoptosis would dominate, causing a decrease in fat weight and size. Additionally, insulin has been shown to be anti-apoptotic [10], possibly limiting the extent to which RSG could cause a predominance of small adipocytes and, hence, an insulin sensitised state.

Although difficult to assess, another possible mechanism that might explain the lack of RSG's effect as an antidiabetic agent in the LIRKO mice is the extremely increased serum insulin concentrations in this model. The lack of the hepatic insulin receptor, with its resultant compensatory beta-cell hyperplasia and defect in insulin clearance results in markedly increased serum insulin concentrations in the LIRKO mouse, which could contribute to desensitisation of insulin action in peripheral tissues.

In conclusion, TZDs could improve some lipid, but not glycaemic, parameters of the dysmetabolic syndrome associated with diabetes mellitus even in the presence of absolute insulin resistance in the liver, whereas for metformin to have any effect, an operating insulin-signalling system in liver is mandatory.

References

1. Kahn CR, Chen L, Cohen SE (2000) Unraveling the mechanism of action of thiazolidinediones J Clin Invest 106: 1305–1307
2. Inzucchi SE, Maggs DG, Spollett GR, Page SL, Rife FS, Walton V, Shulman GI (1988) Efficacy and metabolic effects of metformin and troglitazone in type II diabetes mellitus. N Engl J Med 338:867–872
3. Tontonoz P, Hu E, Spiegelman BM (1994) Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor [published erratum appears in Cell (1995) Mar 24; 80: following 957]. Cell 79:1147–1156
4. Zhou G, Myers R, Li Y et al. (2001) Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 108:1167–1174
5. Michael MD, Kulkarni RN, Postic C et al. (2000) Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. Mol Cell 6:87–97
6. Bruning JC, Michael MD, Winnay JN et al. (1998) A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. Mol Cell 2:559–569
7. Halaas JL, Gajiwala KS, Maffei M et al. (1995) Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269:543–546
8. Vestergaard H, Lund S, Pedersen O (2001) Rosiglitazone treatment of patients with extreme insulin resistance and diabetes mellitus due to insulin receptor mutations has no effects on glucose and lipid metabolism. J Intern Med 250:406–414
9. Chao L, Marcus-Samuels B, Mason MM (2000) Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones. J Clin Invest 106: 1221–1228
10. Tseng YH, Ueki K, Kriauciunas KM, Kahn CR (2002) Differential roles of insulin receptor substrates in the anti-apoptotic function of insulin-like growth factor-1 and insulin. J Biol Chem 277:31601–31611