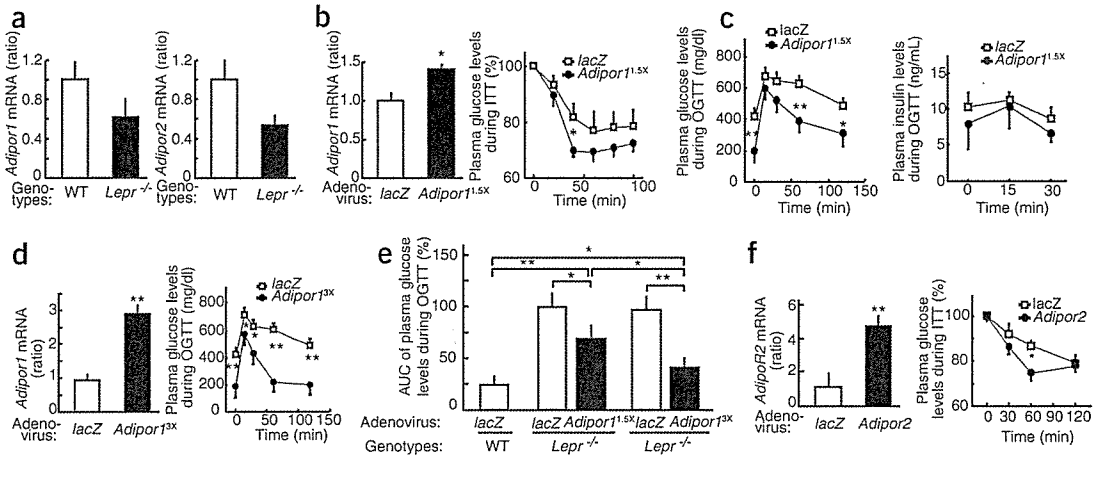

Figure 1 Adenovirus-mediated expression of *Adipor1* or *Adipor2* in the liver of *Lepr*$^{-/-}$ mice improves insulin resistance and ameliorates diabetes. (a–g) The amounts of *Adipor1* and *Adipor2* mRNA in the liver, plasma glucose levels, insulin levels and area under the curves (AUC) during the oral glucose tolerance test (OGTT) or the insulin tolerance test (ITT). We used wild-type C57Bl/6 (WT) and *Lepr*$^{-/-}$ mice. In addition, some mice were infected with an adenovirus expressing *lacZ*, *Adipor1* or *Adipor2*. Adenovirus concentrations used were 1.0×10^8(1.5X) and 3×10^8(3.0X) plaque-forming units (pfu) per gram body weight. Ratios in a,b,d and f are with respect to values of control mice. Percentages in b and f are with respect to values at time 0. Percentage in e is with respect to value of *lacZ*-infected *Lepr*$^{-/-}$ mice. Results indicate mean ± s.e.m. $n = 10$ for wild-type, untreated *Lepr*$^{-/-}$ and AdipoR2-infected *Lepr*$^{-/-}$ mice; 15 for *lacZ*- or R1$^{1.5X}$-infected *Lepr*$^{-/-}$ mice; 11 for R1^{3X}-infected *Lepr*$^{-/-}$ mice. #$P = 0.06$; *$P < 0.05$; **$P < 0.01$ (between two groups, as indicated, or versus *lacZ*).

To determine how decreased expression of *Adipor* mRNA is involved in the development of insulin resistance and diabetes in obese mice, we next studied the effects of restoring *Adipor1* expression, by means of an adenovirus, in *Lepr*$^{-/-}$ mice. Following injection of the adenovirus, *Adipor1* expression levels were increased 1.5-fold in the liver of *Lepr*$^{-/-}$ mice (Fig. 1b), comparable to levels in the wild-type mice (Fig. 1a), which did indeed significantly improve insulin resistance (Fig. 1b) and diabetes (Fig. 1c). However, this improvement was only partial, though significant ($P < 0.01$), since the area under the curve (AUC, a measure of plasma glucose levels during the oral glucose tolerance test) decreased to 74% in *Lepr*$^{-/-}$ mice, compared to an AUC of 24% in the wild-type mice (Fig. 1e). This improvement in diabetes occurred without an increase in plasma insulin levels (Fig. 1c). These data suggest that insulin resistance and diabetes in *Lepr*$^{-/-}$ mice are presumably caused, at least in part, by decreases in *Adipor1*. This notion is further supported by the data showing that even further overexpression of *Adipor1* in the liver of *Lepr*$^{-/-}$ mice (3-fold, Fig. 1d) now markedly ameliorated diabetes (Fig. 1d), indicating an expression level–dependent improvement (Fig. 1e and Supplementary Fig. 1 online). Likewise, adenovirus-mediated 5-fold overexpression of *Adipor2* in the liver of *Lepr*$^{-/-}$ mice (Fig. 1f) markedly improved insulin resistance (Fig. 1f) and ameliorated diabetes (Fig. 1g), and was associated with a decrease in plasma insulin levels (Fig. 1g).

The effects of the adenovirus-mediated 1.5-fold expression of *Adipor1* or 5-fold overexpression of *Adipor2* in the liver on the amelioration of diabetes were lost in adiponectin-deficient[16–19] *Lepr*$^{-/-}$ mice (Supplementary Fig. 1). These data are consistent with our conclusion that the phenotypes resulting from restoration of adiponectin receptors in the liver are due to increased adiponectin signaling.

To identify the physiological mechanisms involved in the amelioration of diabetes seen in our earlier results, we performed a hyperinsulinemic euglycemic clamp experiment (Fig. 2a). Adenovirus-mediated restoration of *Adipor1* significantly reduced endogenous glucose production (EGP), whereas overexpression of *Adipor2* had little effect on EGP (Fig. 2a). However, both adenovirus-mediated restoration of *Adipor1* and overexpression of *Adipor2* increased the glucose infusion rate (GIR) by about 2-fold (Fig. 2a), indicating increased insulin sensitivity.

To further examine the role of *Adipor1* or *Adipor2* in the regulation of gluconeogenesis, we performed a pyruvate challenge test[20] by investigating the rise in plasma glucose in response to the administration of pyruvate, a precursor for gluconeogenesis[20]. Adenovirus-mediated restoration of *Adipor1* significantly suppressed the rise in plasma glucose concentration after pyruvate injection (Fig. 2b), whereas overexpression of *Adipor2* had little effect (Fig. 2b).

AdipoR1 increases AMPK activation by adiponectin in liver

We next studied whether adenovirus-mediated expression of *Adipor1* or *Adipor2* in the liver of *Lepr*$^{-/-}$ mice increases the effects of adiponectin, such as the activation of AMPK (refs. 8,9) and PPAR-α (refs. 10,11). Adenovirus-mediated restoration of AdipoR1 resulted in significantly increased activation of AMPK in the liver by adiponectin (Fig. 2c), whereas overexpression of *Adipor2* did not (Fig. 2d). These results suggested that *Adipor1* may be more involved in the activation of AMPK by adiponectin than *Adipor2* in the liver *in vivo*.

The activation of AMPK in the liver has been reported to reduce the expression of genes encoding hepatic gluconeogenic enzymes such as glucose-6-phosphatase (*G6pc*) and phosphoenolpyruvate carboxykinase 1 (*Pck1*)[21], as well as that of genes encoding molecules involved in lipogenesis, such as sterol regulatory element-binding protein 1c (*Srebf1*)[22]. As predicted by these earlier studies, we found that adenovirus-mediated restoration of *Adipor1* significantly decreased the expressions of *G6pc* (Fig. 2e), *Pck1* (Fig. 2f) and *Srebf1* (Fig. 2g) in the liver of *Lepr*$^{-/-}$ mice; this may be a possible mechanism by which the restoration of *Adipor1* in the liver reduced EGP (Fig. 2a), while

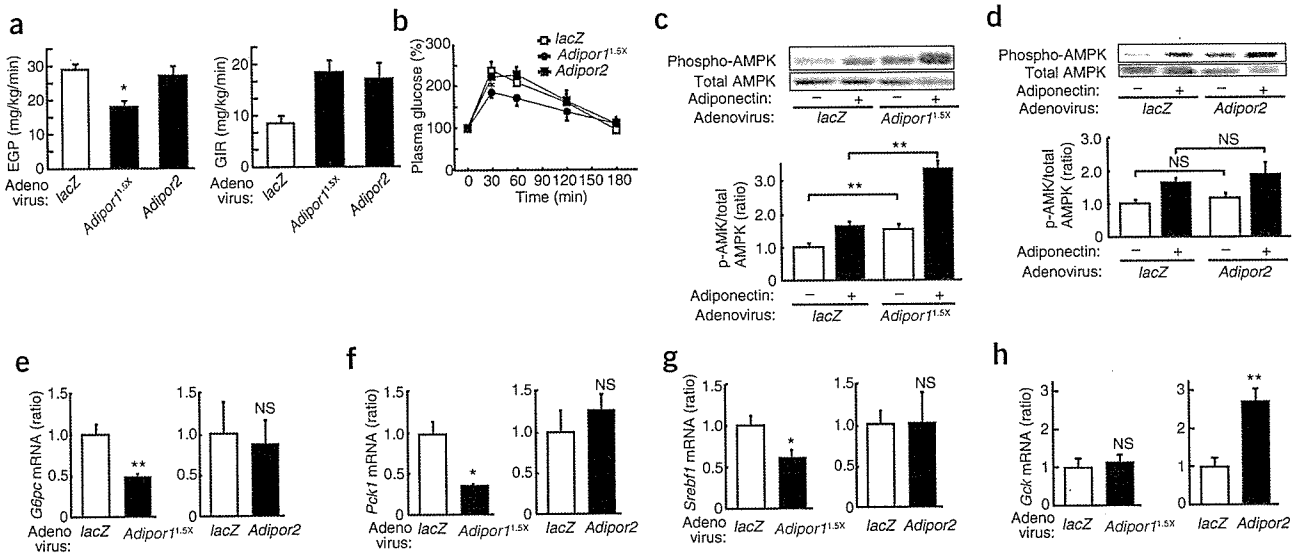

Figure 2 Adenovirus-mediated expression of AdipoR1 in the liver of *Lepr*$^{-/-}$ mice results in activation of AMP kinase pathways. (a) Endogenous glucose production (EGP) and glucose infusion rate (GIR) during hyperinsulinemic euglycemic clamp. (b) Plasma glucose levels during pyruvate challenge test. (c,d) Phosphorylation and amount of AMPK after treatment with or without full-length adiponectin (30 μg per 10 g body weight) for 10 min. (e–h) Amounts of *G6pc*, *Pck1*, *Srebf1* and *Gck* mRNA in the liver of *Lepr*$^{-/-}$ mice infected with an adenovirus expressing *lacZ*, *Adipor1*$^{1.5x}$ or *Adipor2*. Percentage in b is with respect to value at time 0. Ratios in c–h are with respect to values in *lacZ*-infected mice. Densitometric quantification of bands is shown in c and d.; data were corrected for the total amount of AMPK protein in each sample and are expressed as the ratio to the value of vehicle-injected mice infected with adenoviruses expressing *lacZ*. Data are represented as mean ± s.e.m. $n = 7$–12 in b,e–h; 5–7 in a,c,d (per condition). $^*P < 0.05$; $^{**}P < 0.01$; NS, no significant difference (between two groups, as indicated, or versus *lacZ*).

increasing the GIR (Fig. 2a) and improving diabetes (Fig. 1e). In contrast, overexpression of *Adipor2* had little effect on the expression levels of *G6pc* (Fig. 2e), *Pck1* (Fig. 2f) or *Srebf1* (Fig. 2g).

AdipoR2 increases PPAR-α target genes in liver

Although *Adipor2* overexpression had no effect on the gene expression of proteins involved in gluconeogenesis, it did significantly increase the expression of a gene involved in glucose uptake (glucokinase (*Gck*)[23]), whereas restoration of *Adipor1* did not (Fig. 2h). Thus, whereas both *Adipor1* restoration and *Adipor2* overexpression increase the GIR (Fig. 2a) and ameliorate diabetes (Fig. 1c,g) they appear to do so by effects on different aspects of glucose metabolism.

Adenovirus-mediated expression of *Adipor2* in the liver of *Lepr*$^{-/-}$ mice increased the expression of the gene encoding PPAR-α itself (*Ppara*; Fig. 3a) and its target genes[10], including *Acox1* (encoding acyl-CoA oxidase; Fig. 3b) and *Ucp2* (encoding uncoupling protein 2; Fig. 3b). In contrast, adenovirus-mediated expression of *Adipor1* had little effect on any of these genes (Fig. 3a,b). These observations suggested that AdipoR2 may be more involved in the activation of the PPAR-α pathways than AdipoR1. Because both AMPK (ref. 24) and PPAR-α (ref. 10) have been reported to increase fatty acid oxidation, we next examined the effects of adenovirus-mediated expression of *Adipor1* and *Adipor2* on fatty acid oxidation and tissue triglyceride content[25]. We noted a significant increase in the former (Fig. 3c) and a decrease in

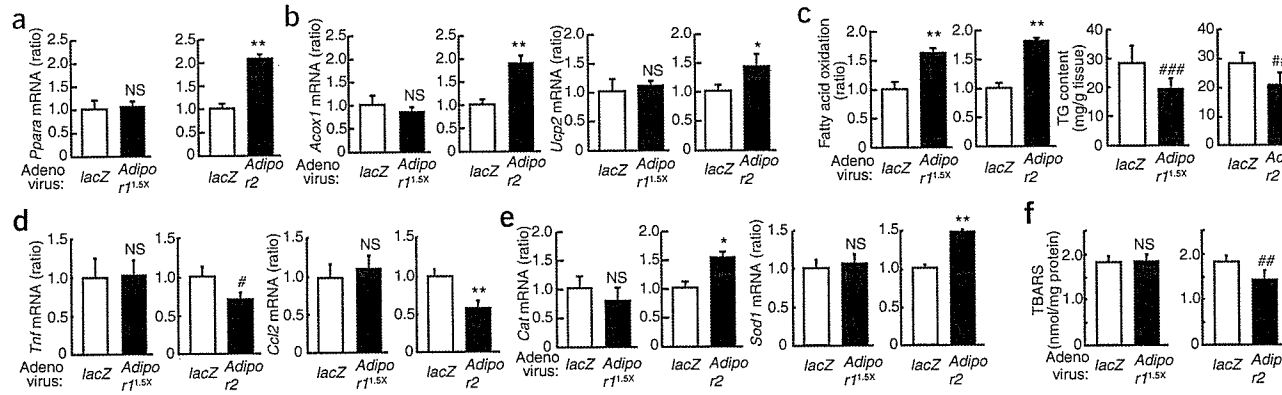

Figure 3 Adenovirus-mediated expression of *Adipor2* in the liver of *Lepr*$^{-/-}$ mice results in activation of PPAR-α pathways. (a,b) Amounts of *Ppara*, *Acox1* and *Ucp2* mRNA in the liver. (c) Extent of fatty acid oxidation and triglyceride content in the liver. (d,e) Amount of *Tnf* and *Ccl2*, and *Cat* and *Sod1* mRNA in the liver. (f) Thiobarbituric acid reactive substance (TBARS) in the liver. *Lepr*$^{-/-}$ mice were infected with an adenovirus expressing *lacZ*, *Adipor1*$^{1.5x}$ or *Adipor2*. Ratios in a–e are with respect to values in *lacZ*-infected mice. Data are represented as mean ± s.e.m. $n = 7$–10 per condition. $^*P < 0.05$; $^{**}P < 0.01$; NS, no significant difference; ###$P = 0.09$ (in c); #$P = 0.12$ (in d); ##$P = 0.11$ (in c,f).

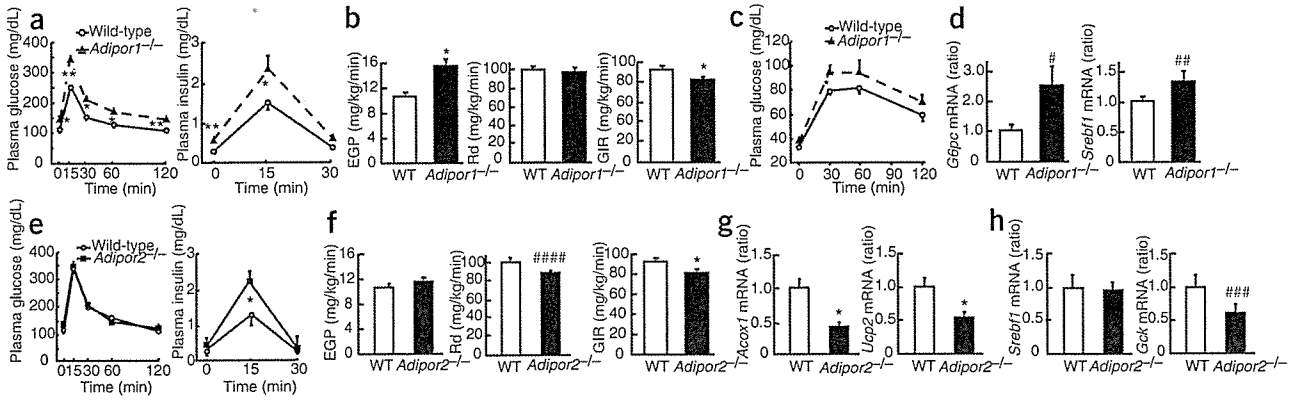

Figure 4 Targeted disruption of *Adipor1* results in increased glucose production, whereas that of *Adipor2* results in decreased glucose uptake. (a) Plasma glucose and plasma insulin in wild-type (WT) and *Adipor1*$^{-/-}$ mice during OGTT (2 g glucose per kg body weight). (b) Endogenous glucose production (EGP), rates of glucose disposal (Rd) and glucose infusion rate (GIR) in wild-type and *Adipor1*$^{-/-}$ mice during hyperinsulinemic euglycemic clamp study. (c) Plasma glucose in wild-type and *Adipor1*$^{-/-}$ mice during pyruvate challenge test. (d) Amounts of *G6pc* and *Srebf1* mRNA in liver from wild-type and *Adipor1*$^{-/-}$ mice. (e) As in a but for in wild-type and *Adipor2*$^{-/-}$ mice. (f) As in b but for wild-type and *Adipor2*$^{-/-}$ mice. (g) Amounts of *Acox1* and *Upc2* mRNA in liver from wild-type and *Adipor2*$^{-/-}$ mice. (h) Amounts of *Srebf1* and *Gck* mRNA in liver from wild-type and *Adipor2*$^{-/-}$ mice. Mice used were from a C57Bl/6 background. Data were obtained during (b,f) or after (d,g,h) the hyperinsulinemic euglycemic clamp studies. Results are stated as mean ± s.e.m. $n = 10$–16 per genotype. $^{*}P < 0.05$; $^{**}P < 0.01$; $\#P = 0.16$ and $\#\#P = 0.15$ (in d); $\#\#\#\#P = 0.07$ (in f); $\#\#\#P = 0.13$ (in h).

the latter (Fig. 3c) as a result of the adenovirus-mediated expression of either *Adipor1* or *Adipor2* in the liver of *Lepr*$^{-/-}$ mice.

Proinflammatory cytokines such as tumor necrosis factor (TNF)-α (ref. 26) and chemokines[14] such as monocyte chemoattractant protein (MCP)-1 (refs. 27–30) are involved in the induction of insulin resistance in obesity, whereas PPAR-α has been reported to reduce it[31–33]. Oxidative stress[15] is also involved in the induction of insulin resistance[34,35], and PPAR-α increases the expression of molecules involved in the reduction of oxidative stress, such as catalase[36] and Cu,Zn-superoxide dismutase (SOD1)[37], whose promoters contain peroxisome proliferator response elements (PPRE). Adenovirus-mediated expression of *Adipor2* in the liver of *Lepr*$^{-/-}$ mice increased *Ppara* (Fig. 3a), reduced *Tnf* and *Ccl2* (the gene encoding MCP-1, also known as chemokine (C-C motif) ligand 2; Fig. 3d), and significantly increased catalase (*Cat*) and *Sod1* (Fig. 3e), while reducing oxidative stress (Fig. 3f). In contrast, adenovirus-mediated expression of *Adipor1* had no statistically significant effects on these

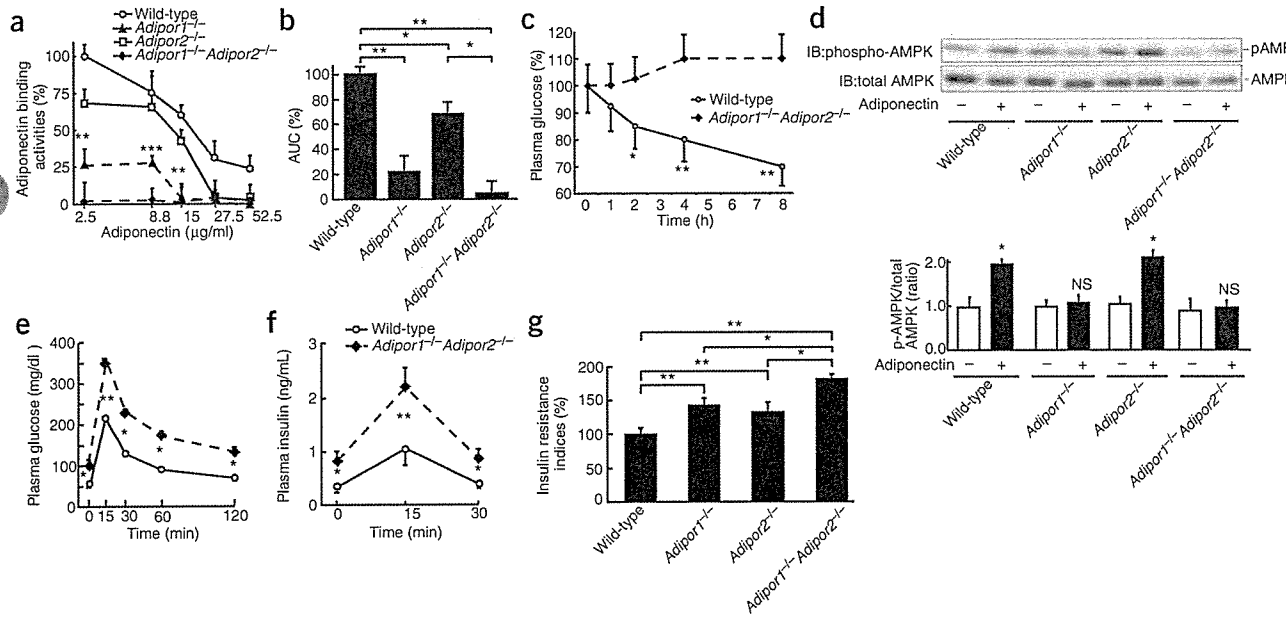

Figure 5 Targeted disruption of both *Adipor1* and *Adipor2* results in abrogation of adiponectin binding and adiponectin actions, leading to marked glucose intolerance and insulin resistance. (a,b) Binding and AUC of binding of full-length adiponectin to the primary hepatocytes. (c) Plasma glucose during the adiponectin sensitivity test (30 μg per 10 g body weight). (d) Phosphorylation and amount of AMPK in liver treated with or without full-length adiponectin (30 μg per 10 g body weight) for 10 min. (e,f) Plasma glucose and plasma insulin during the oral glucose tolerance test. (g) Insulin resistance indices. Mice were on a C57Bl/6 and 129/sv background. Percentages in a and b are with respect to the values in wild-type mice. Percentage in c is with respect to value at time 0. Densitometric quantification of bands is shown in d; data were corrected for the total amount of AMPK protein in each sample and are expressed as a ratio of the values in vehicle-injected wild-type mice. Results are represented as mean ± s.e.m. $n = 6$ in a,b and d; 10 in c; 20 in e and f; and 10-20 in g (per genotype or condition). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P = 0.06$; NS, no significant difference (between two groups, as indicated, or versus wild-type, or versus vehicle).

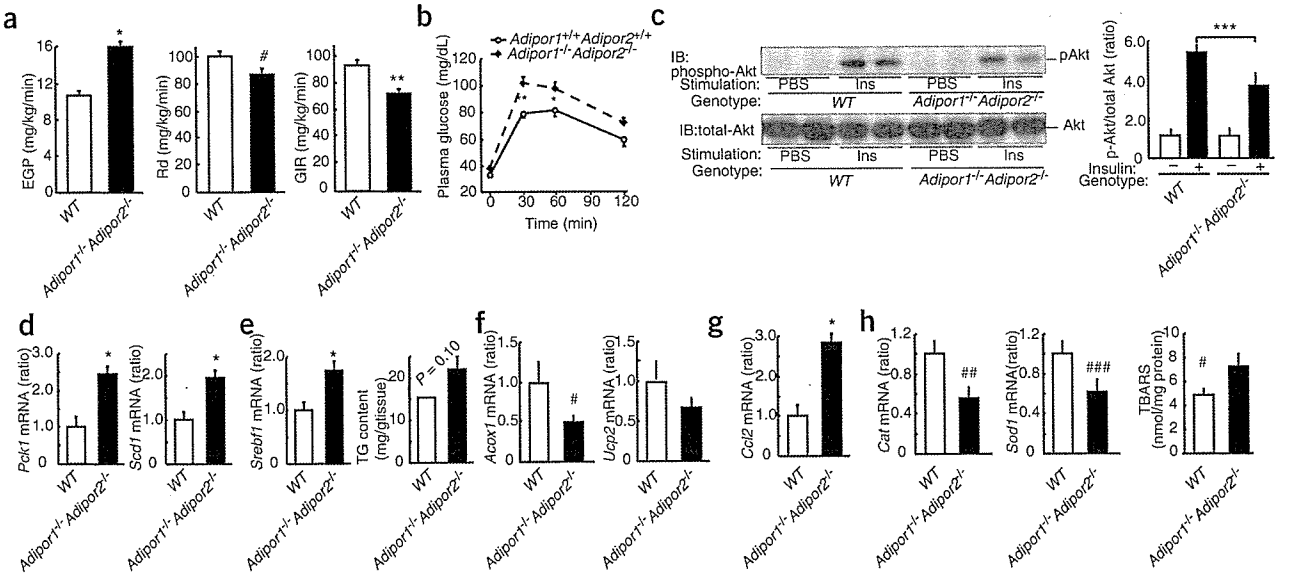

Figure 6 Targeted disruption of both *Adipor1* and *Adipor2* results in dysregulation of AMPK and PPAR-α pathways, leading to increased EGP and decreased GIR. (a) Endogenous glucose production (EGP), rates of glucose disposal (Rd) and glucose infusion rate (GIR) during hyperinsulinemic euglycemic clamp study. WT; wild-type (b) Plasma glucose levels during the pyruvate challenge test. (c) Phosphorylation and amount of Akt treated with or without insulin (0.01 U per g body weight) for 10 min. (d) Amounts of *Pck1* and *Scd1* mRNA. (e) Amount of *Srebf1* mRNA and triglyceride content. (f) Amounts of *Acox1* and *Ucp2* mRNA. (g) Amount of *Ccl2* mRNA. (h) Amounts of *Cat* and *Sod1* mRNA, and TBARS. Data in c–e are from the liver; in f, from skeletal muscle; and in g and h, from white adipose tissue. Mice were on a C57Bl/6 and 129/sv background. Results in c–h are with respect to values in vehicle control (c) or wild type (d–h). Densitometric quantification of bands is shown in c; data were corrected for the total amount of Akt protein in each sample and are expressed as a ratio of the values in vehicle-injected wild-type mice. Results are represented as mean ± s.e.m. $n = 10$ per genotype in a and b; 4–7 per genotype or condition in c–h. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P = 0.07$; $\#P = 0.08$ (in f and h); $\#\#P = 0.10$, $\#\#\#P = 0.06$ (in h) (between two groups, as indicated, or versus wild type).

parameters (Fig. 3a,d–f). The reduction of inflammation and oxidative stress (Fig. 3d–f) also seemed to be among possible mechanisms by which overexpression of *Adipor2* in the liver improved insulin resistance (Fig. 1f,g and Fig. 2a).

Adipor1-KO mice show increased EGP and insulin resistance

We generated *Adipor1*-knockout mice, confirmed by Southern blot analysis (**Supplementary Fig. 2** online). Northern blot analysis and real-time PCR revealed an approximate 50% reduction of *Adipor1* mRNA expression in the liver of heterozygous *Adipor1*-knockout mice (**Supplementary Fig. 2**) and the abrogation of *Adipor1* mRNA expression in the liver, skeletal muscle and white adipose tissue of homozygous *Adipor1*-knockout mice (**Supplementary Figs. 2** and 3 online).

Adipor1-knockout mice were viable and fertile, with normal body weight, and developed normally. Growth curves were similar to those of wild-type mice until at least 1 year of age, and food intake was normal (data not shown). Plasma glucose and insulin levels in fed mice were significantly higher in *Adipor1*-knockout mice relative to control wild-type mice (**Supplementary Fig. 4** online). To assess glucose metabolism in the former group, we performed an oral glucose tolerance test (**Fig. 4a**). *Adipor1*-knockout mice showed significantly impaired glucose tolerance (**Fig. 4a**) and significantly higher plasma insulin concentrations as compared to those in control wild-type mice, indicating that they had developed insulin resistance (**Fig. 4a**). EGP was significantly increased and GIR significantly decreased in *Adipor1*-knockout mice as compared to control wild-type mice (**Fig. 4b**), indicating increased hepatic glucose production and insulin resistance in the former group. Moreover, the pyruvate-induced rise in plasma glucose concentration was significantly increased in *Adipor1*-knockout mice (**Fig. 4c**), suggesting that AdipoR1 is required for the proper regulation of gluconeogenesis. Hepatic *G6pc* expression levels were higher in *Adipor1*-knockout mice than in wild-type mice (**Fig. 4d**), which is consistent with the increased hepatic glucose production in the former (**Fig. 4b**). Hepatic *Srebf1* expression levels were also higher in the knockouts (**Fig. 4d**), consistent with the idea that AdipoR1 is the physiological link between activation of AMPK and adiponectin signaling (**Fig. 2c**).

Adipor2-KO mice show insulin resistance

We also generated *Adipor2*-knockout mice (**Supplementary Figs. 2** and **3**), which were carried to term. Although these mice expressed a small amount of an aberrantly spliced *Adipor2* mRNA, this mRNA could not make a protein (**Supplementary Fig. 2** and **Supplementary Note** online).

Adipor2-knockout mice were viable and fertile (both males and females). Disruption of *Adipor2* did not change body weight. Although glucose intolerance was not observed in these mice (**Fig. 4e**), their plasma insulin levels were significantly higher than in the wild-type mice (**Fig. 4e**), suggesting that they had developed insulin resistance. In contrast to our finding in *Adipor1*-knockout mice, EGP was not significantly higher in *Adipor2*-knockout mice (**Fig. 4f**). However, GIR and glucose disposal were both decreased in these mice (**Fig. 4f**).

To clarify the molecular mechanisms by which AdipoR2 deficiency decreased GIR, we studied the expression levels of molecules involved in glucose metabolism and insulin sensitivity. Hepatic expression levels of PPAR-α target genes, such as *Acox1* (**Fig. 4g**) and *Ucp2* (**Fig. 4g**), were significantly decreased and hepatic *Gck* expression levels slightly decreased (**Fig. 4h**) in *Adipor2*-knockout mice, which may have contributed to decreased GIR in these mice.

Adipor1/r2 KO abrogates adiponectin binding and actions

We also generated *Adipor1/r2* double-knockout mice (**Supplementary Figs. 2** and **3**), which were carried to term. These mice were viable and fertile (both males and females). Simultaneous disruption of *Adipor1* and *Adipor2* did not change body weight.

First, we studied whether adiponectin could specifically bind to primary hepatocytes, a major target of adiponectin action[6,7,19], obtained from each knockout mouse (**Fig. 5a,b**). Specific adiponectin binding to hepatocytes from *Adipor1*-knockout mice was markedly decreased (**Fig. 5a,b**) and that in *Adipor2*-knockout mice was slightly, but significantly, decreased (as assessed by comparing the AUC of binding activities; **Fig. 5b**). Notably, we detected no appreciable adiponectin-specific binding activity in the hepatocytes from *Adipor1/r2* double-knockout mice (**Fig. 5a,b**), indicating undetectable levels of functional adiponectin receptors in hepatocytes from these mice.

We next studied the action of adiponectin in *Adipor1/r2* double-knockout mice. We have previously reported that treating wild-type mice with adiponectin reduces plasma glucose levels and that this was at least partly due to decreased gluconeogenesis resulting from AMPK activation[8]. Here, we found that treatment of wild-type mice with adiponectin significantly reduced plasma glucose levels (**Fig. 5c**), whereas this effect was completely abolished in *Adipor1/r2* double-knockout mice (**Fig. 5c**). These data indicate that AdipoR1 and AdipoR2 serve as the major physiological adiponectin receptors *in vivo*.

Next we examined the role of AdipoR1 and AdipoR2 in adiponectin-induced AMPK activation in the liver *in vivo* (**Fig. 5d**) and in hepatocytes *ex vivo* (**Supplementary Fig. 4**). Adiponectin increased the phosphorylation of AMPK in the liver *in vivo* and in hepatocytes *ex vivo* isolated from wild-type littermates and *Adipor2*-knockout mice but not in those from *Adipor1*-knockout mice and *Adipor1/r2* double-knockout mice (**Fig. 5d** and **Supplementary Fig. 4**). These results demonstrate that AdipoR1, but not AdipoR2, is required for adiponectin-mediated activation of AMPK in the liver.

Adipor1/r2 KO results in marked glucose intolerance

We next studied glucose metabolism in *Adipor1/r2* double-knockout mice. These mice showed markedly impaired glucose tolerance (**Fig. 5e**). Their plasma insulin concentrations were significantly higher than those of control wild-type mice (**Fig. 5f**), indicating that *Adipor1/r2* double-knockout mice exhibit insulin resistance. There was a significant elevation of the insulin resistance index in the double knockouts compared to the *Adipor1*-knockout mice (**Fig. 5g**); this can be attributed to the *Adipor2* deficiency (**Fig. 5g**).

We next performed the oral glucose tolerance test on mice fed a high-fat diet (**Supplementary Fig. 4**). *Adipor1*-knockout, *Adipor2*-knockout and *Adipor1/r2* double-knockout mice exhibited significantly impaired glucose tolerance and increasing plasma insulin levels on a high-fat diet (**Supplementary Fig. 4**), indicating development of insulin resistance and glucose intolerance on this diet.

Adipor1/r2 double-knockout mice showed significantly increased EGP, decreased glucose disposal and significantly decreased GIR (**Fig. 6a**). These observations indicate increased hepatic glucose production and insulin resistance in the liver and potentially decreased glucose uptake and mild insulin resistance in the skeletal muscle. Moreover, the pyruvate-induced rise in plasma glucose concentration was significantly increased compared to wild-type (**Fig. 6b**), almost as much as in *Adipor1* knockout mice (**Fig. 4c**), suggesting that AdipoR1 is required for proper regulation of gluconeogenesis.

In the liver of *Adipor1/r2* double-knockout mice, insulin receptor and insulin receptor substrate(IRS)-2 tyrosine phosphorylation (**Supplementary Fig. 4**) and insulin-stimulated Akt phosphorylation (**Fig. 6c**) were decreased as compared to that in wild-type mice, suggesting insulin resistance in the liver of the double-knockout.

We next studied the expression levels of molecules involved in glucose and lipid metabolism. Simultaneous disruption of both *Adipor1* and *Adipor2* significantly increased the expression levels of genes encoding molecules involved in gluconeogenesis, such as *Pck1* (**Fig. 6d**), which may explain the increased EGP in this strain (**Fig. 6a**). Moreover, simultaneous disruption of both *Adipor1* and *Adipor2* significantly increased the expression levels of genes encoding molecules involved in fatty acid synthesis, such as *Scd1* (ref. 38 and **Fig. 6d**) and *Srebf1* (**Fig. 6e**), while also increasing tissue triglyceride content in the liver (**Fig. 6e**) and decreasing the expression of genes encoding molecules involved in fatty acid oxidation, such as *Acox1* (**Fig. 6f**), and energy expenditure, such as *Ucp2*, in muscle (**Fig. 6f**).

As we had determined for the individual overexpressors of *Adipor1* and *Adipor2*, we examined inflammation and oxidative stress in adipose tissue[39] from knockout mice. Simultaneous disruption of *Adipor1* and *Adipor2* resulted in significantly increased expression of genes encoding chemokines, such as *Ccl2* (**Fig. 6g**), and decreased expression of genes encoding molecules that reduce oxidative stress, such as *Cat* and *Sod1* (**Fig. 6h**), and increased oxidative stress in white adipose tissue (**Fig. 6h**).

Discussion

In this study, we found that expression of *Adipor1* or *Adipor2* in liver can reverse insulin resistance and diabetes in the *db/db* ($Lepr^{-/-}$) mouse model of obesity and type 2 diabetes. The present data suggest that downregulation of *Adipor1* and *Adipor2* in obesity are involved in the development of insulin resistance and diabetes.

We showed the apparent functional differences between AdipoR1 and AdipoR2 in adiponectin signaling pathways. AdipoR1 was more tightly linked to the activation of the AMPK pathways which regulate the inhibition of gluconeogenesis and thus may be associated with the decreased EGP observed in $Lepr^{-/-}$ mice overexpressing *Adipor1* in the liver. In contrast, AdipoR2 was more involved with activation of the PPAR-α pathways, which stimulate energy dissipation and inhibit inflammation and oxidative stress, and thus may be associated with the increased GIR seen in $Lepr^{-/-}$ mice overexpressing *Adipor2* in the liver. Stimulation of fatty acid oxidation by the expression of *Adipor1* and *Adipor2* may be mediated via the AMPK and PPAR-α pathways, respectively, which in turn lead to decreased triglyceride content. Thus, both pathways can increase insulin sensitivity though by different signaling mechanisms. These functional differences between AdipoR1 and AdipoR2 may be accounted for by AdipoR1- or AdipoR2-specific intracellular binding proteins. We are attempting to identify such proteins, which may mediate the activation of AMPK and PPAR-α, respectively, by adiponectin.

In *Adipor1/r2* double-knockout mice, adiponectin-specific binding and adiponectin-induced actions, such as reductions in serum glucose levels, were abrogated. Thus AdipoR1 and AdipoR2 are the major physiological receptors for adiponectin, while mediating most, if not all, parts of adiponectin binding and adiponectin actions *in vivo*. Although T-cadherin[40], which is also expressed in the liver, is reported to be capable of binding to adiponectin, we found that simultaneous disruption of *Adipor1* and *Adipor2* was sufficient to almost completely abolish adiponectin binding to the liver, which is the major organ responsible for mediating the whole-body metabolic effects generated by adiponectin[6,7,19]. Not surprisingly then, the *Adipor1/r2* double-knockout also completely abolished the major adiponectin-induced metabolic effects (such as decreases in serum glucose levels). Thus, it remains to be determined whether T-cadherin is a physiologically relevant receptor, although it may bind adiponectin *in vivo*.

Targeted disruption of either *Adipor1* or *Adipor2* resulted in insulin resistance, and disruption of both resulted in a marked glucose intolerance and insulin resistance. These findings provide the first direct evidence, to our knowledge, that AdipoR1 and AdipoR2 do indeed play important physiological roles in the regulation of insulin sensitivity *in vivo*. However, the observation that the *Adipor1/r2* double-knockout mice appeared to be more insulin resistant and glucose intolerant than the adiponectin-knockout mice raises a few possibilities that we are currently investigating. Namely, there may be other ligands for AdipoR1/AdipoR2 in addition to adiponectin, and AdipoR1 and/or AdipoR2 may have basal constitutive activities.

The insulin resistance in *Adipor1*-knockout mice was manifested in the significant increase in gluconeogenic enzymes, increased EGP and greatly increased plasma glucose during pyruvate challenge tests, which could be accounted for by the abrogation of adiponectin-induced AMPK activation in the liver. In contrast, insulin resistance in *Adipor2*-knockout mice was characterized by the significantly decreased GIR and tendency for decreased glucose disposal, which may have resulted from decreased glucose uptake in the liver and possibly also in the skeletal muscle. It is possible that the decreased glucose uptake in the liver of these mice resulted from a decrease in the number of molecules involved in glucose uptake (such as glucokinase) and/or from a decrease in PPAR-α activation in the liver. It is unlikely that the insulin resistance in the *Adipor1* knockout is due to similar effects because we did not observe decreased PPAR-α activation or decreased glucokinase in that mutant nor was the glucose disposal rate changed. However, we cannot account for the decreased glucose uptake in the skeletal muscle of *Adipor2*-knockout mice by decreased AMPK activation in skeletal muscle because the AMPK pathway does not appear to be regulated by AdipoR2. Indeed, abrogation of adiponectin-induced AMPK activation in muscle was not observed in the *Adipor2*-knockout mice, although it was seen in *Adipor1*-knockout mice (**Supplementary Fig. 5** online). Instead, it may have been due to decreased PPAR-α activation in the skeletal muscle, indirect actions of decreased PPAR-α activation in the liver, increased inflammation, increased oxidative stress in WAT or all of the above. We did not observe any of these alterations in *Adipor1*-knockout mice. To determine the magnitude of insulin resistance and the role of AdipoR2 in skeletal muscle, experiments such as tracer studies, *ex vivo* studies in muscle strips or studies using labeled 2-deoxyglucose injections *in vivo* will be informative and will be the subject of future experiments.

Adipor1/r2 double-knockout mice exhibited the phenotypes of both *Adipor1*-knockout mice and *Adipor2*-knockout mice. Thus they showed significantly increased EGP, decreased glucose disposal and greatly decreased GIR. Both the increase in EGP and the decrease in glucose disposal may be accounted for by *Adipor1* deficiency, which also decreases AMPK activation and increases the levels of gluconeogenic enzymes and increases the rise in plasma glucose during pyruvate challenge test, whereas the *Adipor2* deficiency decreases PPAR-α activation and increases inflammation and oxidative stress. The marked decrease in GIR may be caused by both increased EGP and decreased glucose disposal.

Although relying on different signaling pathways (that is, AMPK and PPAR-α), a common end point (increased fatty acid oxidation and reduced triglyceride content) may be how the expression of either *Adipor1* or *Adipor2* in the liver could improve insulin resistance and diabetes. The reduction of inflammation and oxidative stress also seemed to be among possible mechanisms by which overexpression of *Adipor2* in the liver improved insulin resistance. Moreover, we noted an increase in oxidative stress in white adipose tissue (WAT), which may be one of the mechanisms by which simultaneous disruption of *Adipor1* and *Adipor2* results in marked glucose intolerance, insulin resistance, significantly decreased GIR and slightly decreased glucose disposal.

In conclusion, AdipoR1 and AdipoR2 are key players in the physiological and pathophysiological significance of adiponectin, and are involved in the regulation of glucose and lipid metabolism, inflammation and oxidative stress. This study suggests that agonism of AdipoR1/R2, as well as strategies to increase AdipoR1/R2, may be logical approaches to providing a new treatment modality for insulin resistance and type 2 diabetes linked to obesity.

METHODS

Generation of *Adipor1*- and *Adipor2*-knockout mice. To construct the targeting vector for disruption of the *Adipor1* or *Adipor2* gene, a neomycin resistance gene was substituted for exons 2, 3 and 4 of the *Adipor1* gene or exon 3 of the *Adipor2* gene (**Supplementary Fig. 2**). The strategy for culturing, electroporation of J1 embryonic stem (ES) cells (129/Sv), and screening for homologous recombinant clones was as described previously[16], with slight modifications. Male chimeric mice were mated with C57Bl/6 female mice to generate heterozygous offspring, and F1 progeny from two independently generated male chimeric mice were crossed to obtain F2 mice. Both mice lines showed identical phenotypes in all experiments carried out in this study. *Adipor1*$^{-/-}$/*Adipor2*$^{-/-}$ mice were prepared by *Adipor1*$^{+/-}$/*Adipor2*$^{+/-}$ mouse intercrosses (**Figs. 5** and **6**, and **Supplementary Figs. 2–5**). All experiments in this study were conducted on male littermates. The procedures used for Southern blot analysis have been described previously[16].

Mice. Mice were 8–10 weeks of age at the time of the experiment. They were housed in cages and maintained on a 12-h light-dark cycle. For the experiment depicted in **Supplementary Figure 4**, we used high-fat diet 32 consisting of 25.5% (wt/wt) protein, 2.9% fiber, 4.0% ash, 29.4% carbohydrates, 32% fat and 6.2% water (CLEA Japan Inc.)[30]. For all other experiments, the diets were standard chow (CE-2, CLEA Japan Inc.) with the following composition: 25.6% (wt/wt) protein, 3.8% fiber, 6.9% ash, 50.5% carbohydrates, 4% fat and 9.2% water[18]. Male *Lepr*$^{-/-}$ or C57Bl/6 mice (aged 8–10 weeks) were purchased from Japan CLEA. To study AMPK phosphorylation *in vivo*, we injected 30 μg recombinant murine adiponectin per 10 g body weight intravenously in mice through an inferior vena cava catheter[8]. This resulted in an increase of plasma adiponectin levels, to approximately 30 μg/ml; this showed that the adiponectin dose used in this study was comparable to endogenous adiponectin levels. Mouse full-length adiponectin was generated as previously described[6,8,12]. The glucose tolerance, insulin tolerance and adiponectin sensitivity tests were conducted as previously described[6,8], with slight modifications. For the pyruvate challenge test[20], mice deprived of food overnight were injected intraperitoneally with pyruvate dissolved in saline (2 g/kg). Plasma glucose concentration was measured at the indicated times thereafter. The pyruvate-induced increase in plasma glucose concentration was abolished by previous administration of 3-mercaptopicolinic acid (30 mg/kg), an inhibitor of gluconeogenesis (data not shown), suggesting that this effect of pyruvate was dependent on gluconeogenesis. The animal care and use procedures were approved by the Animal Care Committee of the University of Tokyo.

Northern blot analysis, quantitative analysis by real-time PCR and immunoblotting. Total RNA was prepared from cells or tissues with Trizol (Invitrogen) according to the manufacturer's instructions. We used a real-time PCR method to quantify the mRNAs (ref. 12), with slight modifications (**Supplementary Methods** online). Northern blot analysis was performed as described previously[12,16], with slight modifications. The procedures used for immunoblotting have been described previously[8,18,30]. The livers or muscles were freeze-clamped in liquid nitrogen *in situ*[8,18,30]. Phosphorylation and the protein levels of Akt and AMPKα were determined as described elsewhere[8,18,30]. Representative data from one of two or three independent experiments are shown.

Blood sample assays. Plasma glucose levels were determined using a glucose B-test (Wako Pure Chemical Industries). Plasma insulin was measured with an insulin immunoassay (Shibayagi). Plasma adiponectin levels were determined using a mouse adiponectin ELISA kit (Otsuka Pharmaceutical Co. Ltd.).

Adenovirus-mediated gene transfer *in vivo*. The recombinant adenoviruses Adex1CAAdipoR1 and Adex1CAAdipoR2 were constructed by homologous recombination between the expression cosmid cassette and the parental virus

genome[8]. Mice were injected intravenously with adenoviruses expressing *lacZ*, *Adipor1* (ref. 12) or *Adipor2* (ref. 12), at concentrations of 1×10^8 or 3×10^8 plaque-forming units (pfu) per gram of body weight, as previously described[8]. After 5–7 d, the mice were subjected to an overnight fast before testing. There were no significant differences between the livers from mice injected with adenoviruses expressing *lacZ*, *Adipor1* or *Adipor2*, in terms of the expression levels of housekeeping genes we examined.

Hyperinsulinemic-euglycemic clamp study. Clamp studies were carried out as described previously[18,30], with slight modifications (**Supplementary Methods**).

Mice primary hepatocytes. Hepatocytes were isolated from 8-week-old male mice by the collagenase perfusion method[13], with slight modifications (**Supplementary Methods**).

Binding assay. Recombinant full-length adiponectin was labeled with ^{125}I at Tyr by IODObeads (Pierce) in the presence of Na[^{125}I] (2,000 Ci/mmol, Amersham Pharmacia Biotech) according to the manufacturer's protocol. Binding assay was carried out as described previously[8,12], with slight modifications (**Supplementary Methods**).

Lipid metabolism, lipid peroxidation, and other materials. The measurements of [^{14}C]CO_2 production from [1-^{14}C]palmitic acid were performed using liver slices, as described elsewhere[6,8,11]. Liver homogenates were extracted, and tissue triglyceride content was determined as described previously[6,8,11], with some modifications. To investigate whether oxidative stress was increased, we measured lipid peroxidation, a marker of oxidative injury, represented by plasma thiobarbituric acid reactive substance (TBARS) as described[39]. Briefly, tissue samples were homogenized in a buffer solution containing 50 mM Tris-HCl (pH 7.4) and 1.15% KCl, and then centrifuged. The supernatant was used for the assay. The levels of lipid peroxidation in tissue homogenate were measured in terms of the amount of TBARS using the LPO test (Wako Pure Chemical Industries). All other materials, including chemicals, were from the sources given in refs. 6,8,12,13,18 and 30.

Statistical analysis. Results are expressed as the mean ± s.e.m. Differences between two groups were assessed using unpaired two-tailed *t*-tests. Data involving more than two groups were assessed by analysis of variance (ANOVA).

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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