MKR mice are resistant to the metabolic actions of both insulin and adiponectin: discordance between insulin resistance and adiponectin responsiveness

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Kim, Chul-Hee, Patricia Pennisi, Hong Zhao, Shoshana Yakar, Jeanne B. Kaufman, Kenjiro Iganaki, Joseph Shiloach, Philipp E. Scherer, Michael J. Quon, and Derek LeRoith. MKR mice are resistant to the metabolic actions of both insulin and adiponectin: discordance between insulin resistance and adiponectin responsiveness. Am J Physiol Endocrinol Metab 291: E298–E305, 2006. Most rodent models of insulin resistance are accompanied by decreased circulating adiponectin levels. Adiponectin treatment improves the metabolic phenotype by increasing fatty acid oxidation in skeletal muscle and suppressing hepatic glucose production. Muscle IGF-I receptor (IGF-IR)-lysine-arginine (MKR) mice expressing dominant-negative mutant IGF-IRs in skeletal muscle are diabetic with insulin resistance in muscle, liver, and adipose tissue. Adiponectin levels are elevated in MKR mice, suggesting an unusual discordance between insulin resistance and adiponectin responsiveness. Therefore, we investigated the metabolic actions of adiponectin in MKR mice. MKR and ob/ob mice were treated both acutely (28 μg/g) and chronically (for 2 wk) with full-length adiponectin. Acute hypoglycemic effects of adiponectin were evident only in ob/ob mice but not in MKR mice. Chronic adiponectin treatment significantly improved both insulin sensitivity and glucose tolerance in ob/ob but not in MKR mice. Adiponectin receptor mRNA levels and adiponectin-stimulated phosphorylation of AMPK in skeletal muscle and liver were similar among MKR, wild-type, and ob/ob mice. Thus MKR mice are adiponectin resistant despite normal expression of adiponectin receptors and normal AMPK phosphorylation in muscle and liver. MKR mice may be a useful model for dissecting relationships between insulin resistance and adiponectin action in regulation of glucose homeostasis.

adiponectin resistance; muscle insulin-like growth factor-I receptor-lysine-arginine mouse

Obesity is a well-known risk factor for diabetes, hypertension, and other cardiovascular diseases linked to insulin resistance (24). The insulin resistance of obesity may be related to secretion of a number of cytokines by adipose cells (e.g., leptin, TNF-α, IL-6, resistin, adiponectin) that couple regulation of adiposity with insulin sensitivity. Unlike many adipokines whose levels are negatively correlated with insulin sensitivity, adiponectin has biological effects that mimic the actions of insulin to stimulate glucose uptake (31, 33), increase fatty acid oxidation (6, 33), inhibit hepatic gluconeogenesis (1, 3), and stimulate production of nitric oxide (2). Thus, in humans and rodents, insulin-resistant states, including obesity, diabetes, and cardiovascular diseases, are generally associated with decreased adiponectin mRNA expression and decreased circulating levels of adiponectin (7, 8, 30). Transgenic mice that are homozygous null for adiponectin develop insulin resistance under normal conditions (18) or on a high fat diet (19). Moreover, human mutations of the adiponectin gene resulting in impaired multimerization of adiponectin are linked to increased risk for type 2 diabetes (29). Therapeutic interventions in humans that improve insulin sensitivity result in an increase in adiponectin levels (14–17). Administration of recombinant adiponectin in insulin-resistant, adiponectin-deficient states improves insulin sensitivity (3, 34) and lowers blood glucose levels (1, 23). However, the mechanisms by which adiponectin improves insulin sensitivity and regulation of glucose metabolism are not completely understood.

We recently created the insulin-resistant diabetic muscle IGF-I receptor (IGF-IR)-lysine-arginine (MKR) mouse by transgenically expressing dominant-negative mutant IGF-IRs specifically in skeletal muscle (5). Hybrid formation of mutated IGF-IR with endogenous IGF-IR and insulin receptors causes impairment of both insulin and IGF-I signaling pathways in skeletal muscle. This leads to insulin resistance in fat and liver with rapidly progressive β-cell dysfunction and type 2 diabetes. In contrast with most insulin-resistant rodent models, MKR mice have elevated circulating adiponectin levels when compared with littermate controls (35). Although treatment of MKR mice with PPARγ agonists (thiazolidinediones) causes a further rise in serum adiponectin levels, hyperglycemia and serum insulin levels remain unchanged (13). Because adiponectin may mediate some aspects of thiazolidinedione action (10, 20, 22), elevated adiponectin levels in MKR mice may represent a compensatory response, indicating adiponectin resistance. Therefore, MKR mice are unusual in that they may be resistant to the metabolic actions of both insulin and adiponectin. Investigating effects of acute and chronic adiponectin treatment in MKR mice may provide important new insights into the relationship between regulation of insulin sensitivity and adiponectin action.

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MATERIALS AND METHODS

Production and purification of recombinant murine adiponectin.

Full-length recombinant murine adiponectin was produced in a mammalian expression system, as described previously (1). Briefly, 293-T cells stably transfected with vector pHM1 containing the adiponectin gene were propagated in CD 293 defined medium (Invitrogen, Carlsbad, CA) supplemented with 1-glutamine (2 mM) and vitamin C (100 mg/l). Growth and production took place in a packed bed bioreactor, as described earlier (12). Cells were transferred to production medium in a bioreactor equipped with an internal retention device containing 50 g of Fibra-Cel disks (Bibby Sterlin, Staffordshire, UK) and a vertical mixing system (Celligen Plus; New Brunswick Scientific, Edison, NJ). Bioreactor conditions included dissolved oxygen concentration of 60%, pH 7.0, 37°C, and agitation at 60 rpm. The reactor was perfused with production medium to keep glucose levels constant (5.6 mmol/l). The adiponectin-containing spent medium was concentrated and dialyzed (20 mM Tris pH 8.0, 0.5 mM CaCl₂) using 30,000 molecular weight cut-off regenerated cellulose membranes (PLTK-C 30) (Millipore, Bedford, MA). The concentrated solution was loaded on a Q-Sepharose HP column (GE healthcare) equilibrated in the same buffer. The column was then washed with low-salt buffer (20 mM Tris, pH 8.0, 0.5 mM CaCl₂, 50 mM NaCl) and eluted with a linear gradient from the low-salt buffer to the same buffer containing 0.5 M NaCl. Fractions containing adiponectin were concentrated and dialyzed using PLTK-C 30 to a final adiponectin concentration of 3.0 mg/ml in PBS containing 0.5 mM CaCl₂. The concentrate was centrifuged, and the clear supernatant was filter sterilized. Adiponectin in various fractions was analyzed by immunoblot, and adiponectin concentration was determined using an ELISA kit (Quantikine kit; R&D Systems, Minneapolis, MN). To develop a vehicle control substance for injection, conditioned medium of HEK 293 cells (CRL-1573; ATCC) that did not express the adiponectin gene was subjected to the same purification process described above.

Mouse models. Mice were maintained on a 12:12-h light-dark cycle and were fed NIH-07 rodent food diets (Zeigler Brothers, Gardners, PA) with water available ad libitum. Generation and characterization of MKR mice have been described previously (4, 5). Homozygous MKR male mice (FVB/N background) were used at 6 – 8 wk of age. Wild-type (WT) littermates on FVB/N background at 6 – 8 wk of age. After a 2-wk treatment of MKR mice at 6 – 8 wk old were insulin resistant and diabetic with body weights of 20.7 ± 0.7 g, fasting glucose of 11.0 ± 2.7 mmol/l, and fasting adiponectin of 7.9 ± 1.6 μg/ml, consistent with our previously described metabolic phenotyping. After purification through Q-Sepharose column, we checked the purity of our adiponectin preparation by SDS-PAGE analysis. Protein staining (with SYPRO ruby; Invitrogen) and Western blotting with anti-adiponectin antibody showed that most of the other proteins were removed and adiponectin was enriched (Fig. 1A). To see the multimerization status of the purified adiponectin, we performed SDS-PAGE in nonreduc-
ing, nonheating conditions. As previously reported (27, 29), adiponectin is present in a mixture of trimer, hexamer, and HMW forms (Fig. 1A, lane 1). In heating, reducing conditions adiponectin was reduced to a 30-kDa monomer (Fig. 1A, lane 2). We then checked the bioactivity of purified adiponectin in cultured cells. As already well known (26, 31, 33), adiponectin preparation increased phosphorylation of AMPK in C2C12 myoblasts (Fig. 1B).

Effects of acute adiponectin treatment on glycemia. The effect of acute adiponectin injection (28 μg/g body wt ip) on glycemia in MKR mice was not significantly different from the response to injection of vehicle control (Fig. 2A). To verify that our recombinant adiponectin preparation was biologically active, we repeated these experiments in hyperglycemic ob/ob mice. Consistent with previous reports (1), blood glucose levels 4, 6, 8, and 24 h after adiponectin injection were significantly lower in ob/ob mice when compared with vehicle-injected controls (Fig. 2B). Moreover, in normoglycemic adiponectin knock-out mice, acute injection of adiponectin also lowered blood glucose levels when compared with vehicle-injected controls (data not shown). Thus MKR mice are resistant to the glucose-lowering effects of acute adiponectin treatment.

Metabolic effects of chronic adiponectin treatment. Because MKR mice did not respond to acute adiponectin treatment, we next tested their metabolic response to chronic adiponectin treatment (28 μg·g⁻¹·day⁻¹ ip) for 2 wk. After this chronic treatment, serum adiponectin levels were elevated ~7-fold (Fig. 3A). Levels of the HMW isoforms of adiponectin that may be more bioactive (22) were also significantly increased after 2 wk of adiponectin treatment (Fig. 3B). Despite these marked elevations in serum adiponectin and HMW adiponectin, daily treatment of MKR mice for 2 wk with adiponectin did not significantly alter blood glucose levels (Fig. 3C) or body weight (Fig. 3D) when compared with MKR mice treated with vehicle injections. Moreover, this 2-wk adiponectin treatment did not significantly improve either insulin sensitivity or glucose tolerance in MKR mice (assessed by ITT and GTT) (Fig. 4A and B). By contrast, 2-wk treatment of ob/ob mice with adiponectin significantly improved insulin sensitivity and glucose tolerance when compared with vehicle treatment. Thus, unlike most other insulin-resistant diabetic models, adiponectin treatment of MKR mice does not have beneficial effects to improve insulin sensitivity or glucose metabolism.

Adiponectin signaling in MKR mice. Because MKR mice are resistant to acute and chronic beneficial effects of adiponectin

Fig. 1. Characterization of purified recombinant adiponectin preparation. A: Q-Sepharose column-purified recombinant adiponectin was subjected to SDS-PAGE analysis and then protein staining (by SYPRO ruby; Invitrogen) (left) and Western blotting (WB) with anti-adiponectin antibody (right) in nonreducing (lane 1) and reducing (lane 2) conditions. B: effects of adiponectin treatment on AMP kinase (AMPK) phosphorylation in C2C12 cells. Cells were serum starved overnight and treated with the indicated concentrations of purified adiponectin for 5 min. Total cell lysates were subjected to immunoblotting for phospho-AMPK (pAMPK; top) and total AMPK (bottom). Bar graph shows densitometric quantification of pAMPK normalized for total AMPK. Data are means ± SE of 3 independent experiments. HMW, high molecular weight. *P < 0.001 vs. vehicle control.

Fig. 2. Effects of acute adiponectin treatment (28 μg/g body wt ip) on blood glucose levels in muscle IGF-I receptor-lysine-arginine (MKR; A) and ob/ob (B) mice. Mice were fasted 2 h before treatment and were refed 8 h after the treatment; n = 5 for each group. *P < 0.01 vs. vehicle group by ANOVA.
on insulin sensitivity and glucose metabolism, we next investigated several aspects related to adiponectin signaling in metabolic insulin target tissues. Using quantitative real-time RT PCR, we did not find any significant differences in the levels of mRNA expression for adiponectin receptors R1 and R2 among MKR, WT, and ob/ob mice in either hindlimb skeletal muscle (Fig. 5A) or liver (Fig. 5B). Many biological actions of adiponectin are linked to activation of AMPK. Therefore, we also examined activation of AMPK in response to acute adiponectin injection (28 μg/g body wt ip) in skeletal

Fig. 3. Effects of chronic adiponectin treatment on serum adiponectin concentration (A), %HMW forms of adiponectin (B), blood glucose levels (C), and body weight (D) in MKR mice. Mice were treated with either adiponectin (28 μg·g⁻¹·day⁻¹ ip) or vehicle control for 2 wk; n = 5 for each group.

Fig. 4. Results of insulin tolerance test (insulin 7.5 μU/g ip; A) and glucose tolerance test (glucose 2 mg/g ip; B) after 2 wk of treatment with adiponectin (28 μg·g⁻¹·day⁻¹ ip) or vehicle control in MKR and ob/ob mice; n = 5 for each group. *P < 0.05 vs. vehicle group by ANOVA.
muscle and liver of MKR, WT, and ob/ob mice, as assessed by immunoblotting tissues with a phosphor-specific antibody for AMPK. As expected, acute adiponectin treatment significantly increased phospho-AMPK levels without affecting total AMPK expression in WT mice in both skeletal muscle (Fig. 6A, lanes 1 and 2) and liver (Fig. 6B, lanes 1 and 2). Similar results were observed for ob/ob mice (Fig. 6, A and B, lanes 5 and 6) and adiponectin knock-out mice (data not shown). Interestingly, both basal and adiponectin-stimulated phospho-AMPK levels in skeletal muscle of MKR mice were slightly increased when compared with corresponding results from WT mice (Fig. 6A, lanes 3 and 4). Results in MKR liver samples were similar to those from WT and ob/ob mice (Fig. 6B, lanes 3 and 4). In addition, we have analyzed the phosphorylation status of ACC in skeletal muscle (a single experiment) and liver (at least 2 separate occasions) from WT, MKR, and ob/ob mice. The results showed that in adiponectin-treated mice there is increased phosphorylation of ACC, indicating increased AMPK activity in both skeletal muscle and liver (data not shown). Thus the adiponectin resistance of MKR mice cannot be explained by differences in receptor expression levels or activation of AMPK.

**DISCUSSION**

Adiponectin is a recently described adipokine that can exert systemic effects on insulin sensitivity and lipid metabolism (1, 3, 6, 31, 33, 34). Decreased serum levels of adiponectin are a common feature of obesity and insulin-resistant state in humans and rodents (7, 8, 30). In contrast with most insulin-resistant rodent models, MKR mice have elevated circulating adiponectin levels when compared with littermate controls (35), suggesting resistance to the metabolic actions of adiponectin. The present study confirmed that MKR mice are resistant to metabolic action of adiponectin by showing that these mice did not respond to the dose of adiponectin that significantly lowered blood glucose levels in ob/ob and adiponectin-deficient mice. For our acute adiponectin treatment studies, we withheld food from the animals to maximize the likelihood to observe hypoglycemic effects of adiponectin. One possible explanation for the elevation in adiponectin levels in MKR mice relative to other insulin-resistant models we evaluated is a difference in clearance of adiponectin between the various mouse models. However, even if this were true, it would not explain the resistance of MKR mice to metabolic actions of adiponectin. It seems more likely that the elevated adiponectin levels in MKR mice reflect a compensatory response to adiponectin resistance. Previous studies have shown that adiponectin can lower blood glucose levels in various rodent models. In mouse models of both type 2 (ob/ob) and type 1 (nonobese diabetic or streptozotocin-induced) diabetes,
acute treatment with adiponectin decreases blood glucose levels and hepatic gluconeogenesis (1). In ob/ob mice, adiponectin administration increases thermogenesis, decreases body weight, and reduces serum glucose and lipid levels (23). We confirmed that adiponectin lowers blood glucose levels in ob/ob mice. We also observed that administration of exogenous adiponectin in mice lacking adiponectin lowers blood glucose levels, even though they have normal basal glucose levels. It has been reported that adiponectin knockout mice showed normal basal blood glucose levels despite insulin resistance (18, 19), but it has not been reported that adiponectin treatment further lowers glucose levels in these mice. By contrast, Agouti (A2/a) mice did not respond to intracerebroventricular adiponectin injection in terms of thermogenesis, body weight, and serum glucose (23). There has been no previous report showing resistance to the glucose-lowering effect of peripheral adiponectin injection in mouse models. In this regard, we believe that the MKR mouse is the first mouse model demonstrating peripheral metabolic resistance to adiponectin. It remains possible that MKR mice might respond to even higher doses of adiponectin than we administered. However, these experiments are difficult to perform because of the large amount of recombinant adiponectin that would be required. On the basis of our data, we conclude that doses of adiponectin that are sufficient to significantly raise adiponectin levels and lower blood glucose in several common mouse models of insulin resistance are unable to mediate hypoglycemic effects in MKR mice. Thus it is likely that MKR mice are less sensitive and possibly less responsive to adiponectin despite their significant insulin resistance and diabetes.

Chronic treatment with adiponectin in MKR mice for 2 wk, which increased the serum adiponectin levels ~7 times higher than control, also failed to improve their insulin sensitivity or glucose tolerance. Yamauchi et al. (34) have shown that 2-wk administration of full-length or globular head domain of adiponectin (gAd) reversed insulin resistance associated with both lipoatrophy and obesity. Also, chronic infusion of a very low dose of gAd to mice consuming a high-fat/sucrose diet prevented obesity without affecting food intake (6). We also found that chronic treatment of ob/ob mice with adiponectin significantly improved insulin sensitivity and glucose tolerance. In our chronic treatment experiments, we observed that the fraction of HMW adiponectin was increased disproportionately. Because the HMW form of adiponectin has been implicated as possibly the more bioactive form, these results further confirm that MKR mice are resistant to the metabolic action of adiponectin in improving insulin sensitivity or glucose metabolism, unlike most other insulin-resistant diabetic models.

Two isoforms of adiponectin receptor, AdipoR1 and -R2, have been recently cloned and characterized (32). AdipoR1 is known to be abundantly expressed in skeletal muscle and has high affinity for globular adiponectin, whereas AdipoR2 is predominantly expressed in liver and has intermediate affinity for full-length and globular adiponectin. Adiponectin stimulates the phosphorylation and activation of AMPK in skeletal muscle (26, 33), liver (33), and adipocytes (31), leading to the regulation of glucose metabolism. Activated AMPK inhibits ACC activity, leading to stimulation of fatty acid oxidation and glucose uptake in skeletal muscle and liver. Also in liver, adiponectin reduces expression of enzymes involved in gluconeogenesis, such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, resulting in a decrease of gluconeogenesis. To investigate the mechanism of adiponectin resistance in MKR mice, we checked expression of adiponectin receptors and proximal adiponectin signaling in target tissues. There was no significant difference in the levels of adiponectin receptors AdipoR1/R2 mRNA expression and adiponectin-stimulated phosphorylation of AMPK in skeletal muscle and liver tissues of MKR mice compared with other mice that responded well to adiponectin, such as ob/ob mice. This suggests that the resistance may lie further downstream in the signaling pathways. It is also possible that other signaling molecules regulated by adiponectin, such as Akt and MAPK, may be impaired in MKR mice. However, the major metabolic actions of adiponectin are thought to be mediated by AMPK.

By contrast with our results, Tsuchida et al. (28) reported that AdipoR1/R2 expressions in ob/ob mice were significantly decreased in skeletal muscle and adipose tissue, which was correlated with both decreased adiponectin binding to membrane fractions of skeletal muscle and decreased AMP kinase activation by adiponectin. They suggested that chronic elevation of insulin in obesity could decrease expression of AdipoR1/R2, thereby reducing adiponectin sensitivity, which finally contributes to aggravation of insulin resistance (11). However, they observed adiponectin resistance only in signaling in tissues of ob/ob mice but did not study any effects of adiponectin on whole animal physiology and metabolism. Indeed, Qi et al. (23) reported that ob/ob mice were especially sensitive to adiponectin in vivo, which resulted in increased thermogenesis, weight loss, and reduction in serum glucose and lipid levels, whereas A2/a mice did not respond to adiponectin, indicating that the melanocortin pathway may be a target. It is also controversial as to whether insulin downregulates adiponectin receptors. Inukai et al. (9) reported that insulin increased AdipoR1 expression in C2C12 cells and that AdipoR1 expression was decreased in type 2 diabetic obese db/db mice, but hepatic AdipoR2 expression was not significantly changed. Also, Staiger et al. (25) reported that insulin did not directly modify AdipoR1 expression in human skeletal muscle cells. These discrepancies may be due to the differences in animal model or cell types and conditions used in the studies. More studies will be needed to clarify the role of insulin in regulation of adiponectin receptor expression.

There are a number of possibilities to explain why MKR mice do not respond to adiponectin. First, the elevated adiponectin levels in these mice may be compensatory and already maximal; therefore, the exogenous addition of more adiponectin cannot elucidate an additional effect. Another possibility is that there is adiponectin resistance from a very early phase in MKR mice caused by some yet unknown mechanism that is further exacerbated by insulin resistance. Further studies are therefore needed to elucidate the mechanism of adiponectin resistance in MKR mice.

Adiponectin exists as trimer, hexamer, and HMW forms in both human and mouse plasma (27, 29). It is unclear which adiponectin isoforms bind to each of AdipoR1 and R2 receptors, and it is controversial which form is more biologically active (21, 22, 27, 29). The oligomerization status of our adiponectin preparation was a mixture of various multimer forms, but the HMW form was dominant. Although HMW forms of adiponectin were significantly elevated after injection, they failed to improve glucose metabolism in MKR mice. Thus
the resistance to adiponectin in these mice is probably not related to the changes in oligomerization status. Previous studies have reported that there are differences in biological activity between globular and full-length adiponectin (21). A proteolytic cleavage product of adiponectin that includes its globular head group (gAd) induces free fatty acid oxidation and glucose uptake in muscle (6, 26), whereas full-length adiponectin has been shown to inhibit hepatic glucose production (1, 3). We administered the full-length form of adiponectin. Therefore, it is possible that muscle glucose metabolism was not improved in MKR mice because it may be more responsive to the globular form of adiponectin. However, this hypothesis seems less likely, because we observed stimulation of AMPK phosphorylation in skeletal muscle as well as in liver.

In summary, acute and chronic adiponectin injections failed to improve glucose metabolism in MKR mice, suggesting that these mice are resistant or unresponsive to metabolic actions of both insulin and adiponectin. However, this adiponectin resistance could not be explained by decreased adiponectin receptor gene expression or AMPK phosphorylation in skeletal muscle and liver, suggesting that the resistance may lie further downstream in the signaling pathways. Further work will be necessary to elucidate the molecular mechanism(s) of adiponectin resistance in MKR mice. That should provide novel insights into the role of adiponectin in glucose homeostasis.

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