θ-Defensin RTD-1 improves insulin action and normalizes plasma glucose and FFA levels in diet-induced obese rats

Young Taek Oh, Dat Tran, Thomas A. Buchanan, Michael E. Selsted, and Jang H. Youn

Departments of Physiology and Biophysics, University of Southern California Keck School of Medicine, Los Angeles, California; Department of Pathology and Laboratory Medicine, University of Southern California Keck School of Medicine, Los Angeles, California; Department of Medicine, University of Southern California Keck School of Medicine, Los Angeles, California; and Norris Comprehensive Cancer Center, University of Southern California Keck School of Medicine, Los Angeles, California

Submitted 20 March 2015; accepted in final form 16 May 2015

Oh YT, Tran D, Buchanan TA, Selsted ME, Youn JH. θ-Defensin RTD-1 improves insulin action and normalizes plasma glucose and FFA levels in diet-induced obese rats. Am J Physiol Endocrinol Metab 309: E154–E160, 2015. First published May 19, 2015; doi:10.1152/ajpendo.00131.2015.—Inflammation is implicated in metabolic abnormalities in obesity and type 2 diabetes. Because θ-defensins have anti-inflammatory activities, we tested whether RTD-1, a θ-defensin, improves metabolic conditions in diet-induced obesity (DIO). DIO was induced by high-fat feeding in obese-prone CD rats from 4 wk of age. Starting at age 10 wk, the DIO rats were treated with saline or RTD-1 for 4 or 8 wk. RTD-1 treatment did not alter body weight or calorie intake in DIO rats. Plasma glucose, FFA, triglyceride (TG), and insulin levels increased in DIO rats; RTD-1 normalized plasma glucose and FFA levels and showed tendencies to lower plasma insulin and TG levels. Hepatic and skeletal muscle TG contents increased in DIO rats; RTD-1 decreased muscle, but not hepatic, TG content. Insulin sensitivity, estimated using homeostasis model assessment of insulin resistance and the glucose clamp technique, decreased in DIO rats, but this change was markedly reversed by RTD-1. RTD-1 had no significant effects on plasma cytokine/chemokine levels or IL-1β and TNF-α expression in liver or adipose tissues. RTD-1 treatment decreased hepatic expression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, suggesting that the effect of RTD-1 on plasma glucose (or insulin action) might be mediated by its effect to decrease hepatic gluconeogenesis. Thus, RTD-1 ameliorated insulin resistance and normalized plasma glucose and FFA levels in DIO rats, supporting the potential of RTD-1 as a novel therapeutic agent for insulin resistance, metabolic syndrome, or type 2 diabetes.

high-fat feeding; inflammation; insulin resistance; type 2 diabetes; gluconeogenesis

θ-defensins are naturally occurring, cyclic octadecapeptides expressed in Old World monkeys but not in great apes or humans (26, 30, 31). They are the only known class of cyclic peptides of animal origin (5). Selsted et al. (34) have shown that θ-defensins have potent anti-inflammatory effects in vitro and in vivo, in contrast to proinflammatory and immune-activating properties of mammalian α- and β-defensins, genetically related to θ-defensins (1, 11). The macrocytic conformation of θ-defensins confers remarkable stability in vivo and in vitro (5). Preclinical studies have shown the peptides to be nontoxic, nonimmunogenic, and nonimmunosuppressive at doses that inhibit inflammation in vivo (25), providing strong support for their potential as anti-inflammatory therapeutics. Although the details of the mode of action are still being investigated, it is evident that the lead molecule rhesus θ-defensin 1 (RTD-1) suppresses Toll-like receptor (TLR)-mediated secretion of several pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 (25).

Circulating levels of proinflammatory cytokines are increased in obesity (8). Inflammatory cytokines can induce insulin resistance (27) and may have direct effects on pancreatic β-cells (9, 35). Elevated levels of IL-1β, IL-6, and C-reactive protein are implicated in the pathogenesis of type 2 diabetes (8). Inflammatory cytokines are produced by macrophages that infiltrate adipose tissue in obesity (33, 38). This effect may be particularly prominent in visceral adipose tissue (3). Strategies to mitigate this inflammation include blockade of the cytokines/chemokines or their receptors that mediate macrophage infiltration [e.g., CC-chemokine ligand 2 (CCL2; also known as MCP1) and its receptor, CCR2 (3, 32)] and inhibition of cellular inflammatory responses [e.g., IKKβ and NF-κB by salsalate (10) or IL-1β by monoclonal antibodies or antagonists (8)]. To date, CCL2/CCR2 blockade has not resulted in significant effects to ameliorate insulin resistance (7), possibly due to the redundancy between multiple macrophage chemotactic factors (8, 14). Salsalate had very small effects on lowering A1C in people with type 2 diabetes (12). On the other hand, IL-1β blockade shows greater promise (18, 19). However, IL-1β blockade using monoclonal antibodies is likely to suffer from immunosuppressive side effects observed with TNF blockers (23). Thus, other anti-inflammatory approaches should be investigated. The present study was designed to test whether RTD-1 ameliorates insulin resistance or improves metabolic parameters in DIO.

materials and methods

RTD-1. The hydrochloride salt of synthetic RTD-1 (>98%) was prepared as described previously (30), dissolved in endotoxin-free diluent (0.9% NaCl) at 4 mg/ml, and stored at 4°C.

Induction of DIO and treatment with RTD-1. Obese-prone CD rats were purchased from Charles River at the age of 4 wk. The animals were housed under controlled temperature (22 ± 2°C) and lighting (12-h light, 6 AM–6 PM; 12-h dark, 6 PM–6 AM) with free access to water and food. The animals were fed either a high-fat (90%) diet (HFD; Harlan Teklad, TD.06414) or normal rat chow [low-fat (12%) diet; LFD] for up to 14 wk. The animals on HFD received either saline [0.9% NaCl] or normal saline (in matched volume and frequency) treatment after 6 wk of high-fat feeding (n = 8 for each treatment). The RTD-1 and saline treatments were continued for 4 or 8 wk while
animals were maintained on HFD. The animals on LFD (n = 8) received neither saline nor RTD-1 injections. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Southern California.

Tail catheterization for blood sampling and hyperinsulinemic-euglycemic clamps. At least 4 days before blood sampling and euglycemic clamps (see Experimental protocols), animals were placed in individual cages and accommodated to tail restraints, as previously described (16, 17), required to protect tail blood vessel catheters during the experiments. The animals were free to move about and were allowed unrestricted access to food and water. One tail vein infusion catheter was placed the day before the experiment, and one tail artery blood sampling catheter was placed the morning of the experiment (~7 AM).

Experimental protocols. Experiments were conducted in conscious states, starting at ~1 PM, after a 6-h fast. Blood samples were obtained using the tail artery catheter for basal plasma levels of glucose, insulin, FFA, TG, and cytokines/chemokines. Blood samples were rapidly spun, and plasma samples were aliquoted, frozen immediately in liquid N2, and stored at −70°C for later analysis. Subsequently, a 2-h hyperinsulinemic-euglycemic clamp was conducted by infusing human insulin (5 mU·kg−1·min−1 Novolin;Novo Nordisk, Princeton, NJ) to raise plasma insulin in physiological ranges (16, 17). Plasma glucose was monitored at 10- to 20-min intervals, and 20% dextrose solution was infused to maintain plasma glucose at basal levels (~140 mg/dl). Glucose infusion rates required during the clamp represent insulin’s action to inhibit hepatic glucose output and increase peripheral glucose uptake. At the end of the clamp periods, the animals were euthanized by an intravenous injection of pentobarbital = 0.023

RESULTS

Food intake and body weight. High-fat feeding in obese-prone CD rats resulted in expected increases in body weight compared with those fed LFD (Fig. 1A). RTD-1 treatment did not significantly alter body weight in HFD-fed rats compared

**Fig. 1.** Changes in body weight (A and B) and calorie intake (C and D) during feeding and drug treatments. B and D: data for week 14. Data are means ± SE for 4 or 8 rats.
with the saline-treated group ($P > 0.05$; Fig. 1, A and B). HFD markedly increased fat tissue in the abdominal cavity, and this was not significantly reduced by RTD-1 treatment. RTD-1 treatment showed a tendency to decrease calorie intake during high-fat feeding, but this effect did not gain statistical significance ($P = 0.102$; Fig. 1, C and D).

**Plasma glucose, FFA, TG, and insulin levels.** At the end of 4-wk or 8-wk treatments with RTD-1 or saline, changes in plasma glucose, TG, FFA, and insulin (and other data), induced by high-fat feeding or RTD-1 treatment, were identical between the 4-wk- and 8-wk-treated groups, and the two sets of data were combined for analysis. Compared with LFD, HFD significantly increased basal plasma glucose in the absence of RTD-1, but this increase was completely prevented by RTD-1 treatment in the parallel HFD group (Fig. 2A). High-fat feeding showed a tendency to increase basal plasma FFA levels (by 20%); this increase was completely prevented by RTD-1 treatment (Fig. 2B). Basal plasma TG and insulin levels were significantly raised by high-fat feeding (Fig. 2, C and D). RTD-1 decreased plasma TG and insulin levels in HFD-fed rats, but these effects did not gain statistical significance.

**Liver and muscle TG levels.** HFD increased hepatic TG levels ninefold compared with rats maintained on LFD (Fig. 3A). RTD-1 treatment had no effect on hepatic steatosis in HFD-fed rats. High-fat feeding also increased muscle TG levels, and RTD-1 treatment decreased muscle TG levels with a marginal statistical significance (Fig. 3B).

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**Fig. 2.** Plasma glucose (A), FFA (B), TG (C), and insulin (D) levels in the 3 experimental groups. Blood samples were collected after a 6-h fast. Data are means ± SE for 7 or 8 rats.

**Fig. 3.** Liver (A) and muscle (B) TG levels in the 3 experimental groups. Data are means ± SE for 7 or 8 rats.
Insulin resistance estimated by HOMA-IR was significantly increased by high-fat feeding (Fig. 4). The increase in HOMA-IR was markedly (59%) reversed by RTD-1 treatment ($P = 0.04$), indicating a significant insulin-sensitizing effect.

Insulin sensitivity measured by the glucose clamp. During glucose clamp studies, plasma glucose concentrations (Fig. 5A) were well matched among the three study groups. Plasma insulin concentrations (Fig. 5B) were raised to similar levels in HFD-fed rats with vs. without RTD-1; these level were higher than those in LFD-fed rats, but increases from baseline were not statistically different among the groups, due in part to the higher fasting insulin levels in HFD groups. Glucose infusion rates (GINF) required during the clamp (i.e., during insulin infusion) represent insulin’s action to inhibit hepatic glucose output and increase peripheral glucose uptake. GINF was substantially lower in the HFD group than in the LFD group (Fig. 5, C and D). RTD-1 treatment increased GINF by 56% in HFD rats, reversing insulin resistance by 46%, consistent with the HOMA data.

Plasma and adipose tissue cytokine levels. Of 27 cytokines that can be detected by the Rat Cytokine/Chemokine Multiplex Kit from Millipore, only 10 were measured reliably (i.e., SE < 20% in all groups; Table 1). Plasma levels of leptin, IL-4, VEGF, and CCL11 (eotaxin) were significantly increased by high-fat feeding; RTD-1 treatment did not reverse these changes. Other cytokines, such as CCL5 (RANTES), CXCL5 (LIX), CX3CL1 (fractalkine), IL-5, IL-12 p70, and MCP-1, were affected neither by high-fat feeding nor by RTD-1. The kit did not have sufficient sensitivity to detect TNF-α or IL-6 in our basal plasma samples and produced too much variation for IL-1β, so we do not know whether plasma levels of these cytokines were affected by HFD or RTD-1. We next measured the expressions of IL-1β, IL-6, and TNF-α in epididymal adipose tissue and liver using qRT-PCR. Figure 6 shows that neither IL-1β nor TNF-α showed changes in gene expression in liver or adipose tissue with HFD or RTD-1 treatment ($P > 0.05$). IL-6 expression levels were too low to be accurately quantified (data not shown).

DISCUSSION

The present study demonstrates that RTD-1, a θ-defensin, improves insulin action and normalizes plasma glucose and FFA levels in DIO rats. These effects occurred without altering

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**Fig. 4.** Homeostasis model of assessment of insulin resistance (HOMA-IR) for the 3 experimental groups. Data are means ± SE for 7 or 8 rats.

**Fig. 5.** Plasma glucose (A), insulin (B), and glucose infusion rate (GINF; C) during a hyperinsulinemic-euglycemic clamp. D: steady-state (final 30 min) GINF. Data are means ± SE for 7 or 8 rats.
body weight, food intake, or fat accumulation in the liver. Interestingly, RTD-1 treatment altered neither plasma cytokine/chemokine levels nor adipose or hepatic cytokine expression, and it is unclear whether the RTD-1 effects on insulin action, plasma glucose, and plasma FFA relate to its anti-inflammatory properties (see below). RTD-1 did reduce fat in skeletal muscle and lowered liver expression of two important enzymes for gluconeogenesis. Although the contribution of these changes to the effects of RTD-1 are unclear, our results support the potential of RTD-1 (and possibly other 8-defensins) as a new class of therapeutics for insulin resistance and related disorders such as type 2 diabetes.

Human studies on the natural history of type 2 diabetes showed that insulin resistance occurs long before the development of hyperglycemia (2). Normally, euglycemia can be maintained as β-cells secrete more insulin to compensate for insulin resistance. Blood glucose increases when β-cell function (or compensation) becomes impaired (2, 37). Amelioration of insulin resistance with insulin-sensitizing thiazolidinedione drugs can arrest β-cell decline (4, 36), suggesting that insulin resistance is a cause of failing β-cell function that leads to hyperglycemia. Increased fasting glucose in HFD rats treated with saline suggests inadequate compensation for insulin resistance in those animals. The full reversal of fasting hyperglycemia by RTD-1 despite only partial amelioration of insulin resistance in HFD rats suggests additional mechanisms that cannot be determined from the present study. However, the reduction in insulin levels observed following RTD-1 may have biological importance. The strongest predictor of prevention of diabetes with thiazolidinediones was an initial reduction in insulin levels (4, 36), reflecting reduced secretory demands on β-cells. To the extent that RTD-1 reduces secretory demands on β-cells, it holds promise as an intervention that can alter the natural history of the β-cell disease that leads to hyperglycemia in type 2 diabetes.

Insulin sensitivity was assessed in the present study by HOMA-IR, which is based on basal glucose and insulin levels, and G_{INF} required during a glucose clamp. The two independent methods agreed well to indicate that insulin resistance in DIO rats was 50% reversed by RTD-1 treatment. However, these methods do not disclose whether insulin action was improved in the liver (to decrease hepatic glucose production), peripheral tissues (to increase glucose uptake), or both. Regarding this issue, muscle TG was significantly increased by high-fat feeding, but this change was prevented by RTD-1 treatment. It is well established that accumulation of TG in muscle fibers is associated with peripheral insulin resistance (15, 28). Therefore, RTD-1-mediated improvement of whole body insulin action may be mediated, at least in part, by normalizing TG content (and thus insulin action) in skeletal muscle. In addition, hepatic expression of major gluconeogenic genes was decreased by RTD-1 treatment (Fig. 7), which would lead to improved insulin action to suppress hepatic glucose production. Thus, RTD-1 might improve insulin action both in the liver and in peripheral tissues in DIO rats. The mechanisms by which RTD-1 treatment decreased muscle TG content are unclear; this effect may relate to RTD-1’s effects to lower plasma FFA levels, but the possibility that RTD-1...
increased lipid oxidation in skeletal muscle cannot be excluded. RTD-1 treatment showed a tendency to decrease caloric intake, and this effect may play a role in lowering plasma FFA or muscle TG levels. The effects of RTD-1 to decrease hepatic expression of gluconeogenic genes were associated with no effect on fatty liver. These data are consistent with previous suggestions that there is a strong dissociation between fatty liver and hepatic glucose production or insulin resistance (29), and inhibition of hepatic gluconeogenesis can improve hepatic insulin resistance but also lead to fatty liver (13).

Our data show impressive effects of RTD-1 to completely normalize basal blood glucose and FFA levels in DIO rats. This is in contrast to only a partial reversal of insulin resistance with RTD-1 treatment. The effects on blood glucose and FFA levels might arise, at least in part, from hormonal changes in the basal state. There is a set of hormones, such as catecholamines, glucagon, and cortisol (corticosterone in rodents), that increase both glucose and FFA levels by stimulating hepatic glucose production and lipolysis, respectively. If these hormone levels are decreased in DIO rats by RTD-1, such effects would explain normalization of plasma glucose and FFA. The finding that RTD-1 decreased hepatic expression of gluconeogenic genes supports this idea. In addition, because these hormones are known to induce insulin resistance (6, 24), such effects could also explain the improvement of insulin resistance by RTD-1 treatment.

Schaal et al. (25) demonstrated anti-inflammatory effects of θ-defensins in vitro and in vivo. θ-Defensins suppressed pro-inflammatory cytokine/chemokine release in vitro in peripheral blood leukocytes and cultured THP-1 monocytes stimulated by various TLR agonists. In addition, RTD-1 treatment suppressed rises of proinflammatory cytokines in vivo in mice challenged with bacterial infection. Thus, anti-inflammatory properties of θ-defensins were demonstrated with inflammatory stimuli or bacterial infections. In contrast, RTD-1 treatment did not alter cytokine/chemokine levels in uninfected (i.e., unstimulated) animals. In the present study, RTD-1 treatment did not significantly alter the plasma cytokine/chemokine levels that we measured in DIO rats, as the animals were studied in basal (i.e., unstimulated) states, and inflammation in these animals, if any, was very mild, indicated by the changes in plasma cytokine/chemokine levels (Table 1).

A previous study by Cao et al. (21) showed that human neutrophil α-defensins (HNP-1) reduced hepatic expression of gluconeogenic enzymes (i.e., PEPCK and G6Pase), lowered blood glucose, and improved insulin action, similar to the RTD-1 effects observed in the present study. Whereas θ-defensins have anti-inflammatory properties, several α-defensins, including HNP-1, have proinflammatory properties (1). Similar effects of RTD-1 and HNP-1 suggest the intriguing possibility that the RTD-1 effects arise from structural features shared by α- and θ-defensins [e.g., six conserved cysteines, three intramolecular disulfide bonds, β-sheet regions, etc. (20)] rather than RTD-1’s anti-inflammatory properties. This is supported by the finding that RTD-1 had no significant effects on plasma cytokine levels or IL-1β and TNF-α expression in liver and adipose tissue. Future studies are warranted to address this important issue.

ACKNOWLEDGMENTS

J. Youn is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

GRANTS

This work was supported by ADA Basic Science Award No. 7-12-BS-214 and NIH CTSI Pilot grant (to J. H. Youn), AI-022931, DE-021341, and Arthritis Foundation No. 5997 (to M. Selsted), and UL1 TR-000130 (to T. A. Buchanan).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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