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A retinoid X receptor antagonist, HX531, improves leptin resistance without increasing plasma leptin level in KK-A^y mice under normal dietary conditions

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Abstract

4-(5*H*-2,3-(2,5-Dimethyl-2,5-hexano)-5-methyl-8-nitrodibenzo[b,e][1,4]diazepin-11-yl)benzoic acid (HX531) is a novel retinoid X receptor antagonist. This study provides evidence that HX531 improves leptin resistance without increasing plasma leptin levels in KK-A^y mice, an animal model with high plasma leptin levels and leptin resistance. Under normal dietary conditions, 3 weeks of treatment with HX531 (0.03% and 0.06% food admixture) in KK-A^y mice decreased plasma leptin levels in a dose- and time-dependent manner, in addition to decreasing body weight and mesenteric fatty tissue weight. To evaluate the effect of HX531 on leptin resistance, leptin was injected intraperitoneally in the KK-A^y mice for 4 days after 1 week of treatment with HX531 (0.06% food admixture). This pretreatment with HX531 resulted in exogenously administered leptin causing a significant decrease in food intake. These results suggested that HX531 decreased plasma leptin levels accompanied by a decrease in fatty tissue content in the KK-A^y mice and a simultaneous improvement in leptin resistance. This is the first report that HX531 improves leptin resistance without increasing plasma leptin level in KK-A^y mice, under normal dietary conditions.

1. Introduction

Leptin, the product of the obese gene, is an adipocyte-derived blood-borne satiety factor that decreases food intake and increases energy expenditure, thereby leading to marked reductions in body weight [1-3]. The potent biologic effects of leptin would be expected to be useful in the treatment of obesity and related metabolic disorders and therefore has attracted the interest of many investigators. However, a number of studies in models of rodent and human obesity have demonstrated that plasma leptin concentrations are elevated in proportion to the degree of adiposity, suggesting a state of "leptin resistance" in obesity [4-9]. The KK-A^y mouse is a rodent model of leptin resistance that develops maturity-onset obesity with hyperleptinemia [10]. In a preliminary study, we found food intake in KK-A^y mice was not decreased by leptin administered exogenously at a dosage that reduced food intake in normal mice (ICR mice).

To regulate transcription, the peroxisome proliferator–activated receptor γ (PPARγ), a ligand-activated transcription factor and a member of the nuclear hormone receptor superfamily, binds preferentially to DNA as a heterodimer with a common partner, retinoid X receptor (RXR) [11-13]. It is well established that PPARγ/RXR activation increases insulin sensitivity [14,15]. In 1999, 4-(5*H*-2,3-(2,5-dimethyl-2,5-hexano)-5-methyl-8-nitrodibenzo[b,e][1,4]diazepin-11-yl)benzoic acid (HX531), a dibenzodiazepine derivative was identified as a synthetic RXR antagonist [16]. Recently, it was reported that 1 week of treatment with HX531 in obese KK-A^y mice [17] increased serum leptin levels and potentiated the effects of the hormone, leading to a decrease in food intake and body weight. This very interesting data suggested that control of PPARγ/RXR activity by the RXR antagonist had the beneficial effect of improving leptin resistance. However, this study was carried out under uncomplicated animal breeding conditions, in which the KK-A^y mice with leptin resistance were fed a high-fat diet. This study also did not clarify whether HX531 improved leptin sensitivity of KK-A^y mice under normal dietary conditions as a consequence of increased levels of plasma leptin.

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The aim of the present study was therefore to determine whether HX531 improved leptin resistance in KK-A^y mice fed a normal diet. We report that HX531 treatment for 3 weeks decreased plasma leptin levels in these mice. We also showed that leptin administered exogenously decreased food intake in KK-A^y mice after pretreatment with HX531. Taken together, these findings provide evidence that HX531 improves leptin resistance by increasing leptin sensitivity and not by increasing plasma leptin levels in KK-A^y mice under normal dietary conditions.

2. Materials and methods

2.1. Materials

HX531 was synthesized at the Chemical Research Laboratories of Nissan Chemical Industries (Funabashi, Japan).

Recombinant mouse leptin (Genzyme/TECHNE, Minneapolis, Minn) was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan).

2.2. Effects of 3 weeks of HX531 treatment in KK-A^y mice

Male, 9-week-old KK-A^y mice (CLEA Japan, Tokyo, Japan), a murine model of type 2 diabetes, were used in the study. The mice were divided into 3 groups based on plasma glucose levels: (1) control (n = 4), (2) 0.03% HX531 (n = 5), (3) 0.06% HX531 (n = 4). Mice were fed powdered chow (CMF powder, Oriental Yeast Co Ltd, Tokyo, Japan), whereas HX531 was provided as a food admixture for 3 weeks at the percentages listed above. Body weight and food intake were measured gravimetrically every 2 days whereas rectal temperature of the mice was measured every week. Blood samples were also collected every week from the orbital vein plexus using a capillary pipette. Plasma glucose levels were determined using a glucose analyzer II (Beckman, Fullerton, Calif), plasma leptin levels by an enzyme-linked immunosorbent assay–based mouse leptin immunoassay kit (GT, Minneapolis, Minn), and plasma 3-hydroxy-butylate (3-HB) levels by a test kit (3-HB Kinos, Kinos, Tokyo, Japan). At the end of the experiment, the mice were killed and the mesenteric fatty tissue collected and weighed gravimetrically.

2.3. Food intake restriction study in KK-A^y mice

Male, 9-week-old KK-A^y mice were used. The mice were divided into 2 groups based on plasma glucose levels: (1) control (n = 5) and (2) 50% cellulose (n = 5). Mice were fed powdered chow (CMF powder), whereas 50% cellulose was provided as a food admixture for 3 weeks. Body weight and food intake were measured gravimetrically every 2 days. Blood samples were collected from the orbital vein plexus using a capillary pipette at the final day of experiment period. Plasma leptin level was determined by an enzyme-linked immunosorbent assay kit. In the animals given 50% cellulose food admixture, food intake was calculated as control powdered chow contents in the 50% cellulose admixture mice fed.

2.4. Assay of leptin sensitivity

Male, 9-week-old KK-A^y mice were divided into 2 groups based on plasma glucose levels. In both groups, HX531 was provided as a food admixture (0.06%, vol/vol) for 1 week, followed by daily intraperitoneal (IP) injections for 4 days of either leptin (5 μg/g body weight per day, n = 5) or vehicle (saline, n = 5). Throughout the period of leptin or vehicle administration, control powdered chow (CMF powder) was provided to the mice, with food intake being measured to assess the effects of leptin administration.

2.5. Statistical analyses

All results are expressed as the mean ± SEM. Statistical analysis was performed on a Macintosh computer using either Super Anova version 1.11 (Abacus Concepts, Berkeley, Calif) or Stat View-J 4.5 (Abacus Concepts). Dunnett multiple test was used for multiple comparisons, whereas Student *t* test was applied for comparisons between the 2 groups. Probability values less than .05 were considered as statistically significant.

3. Results

3.1. Effects of HX531 on body weight, rectal temperature, plasma 3-HB and leptin levels, and mesenteric fatty tissue weight in KK-A^y mice

In the group administered 0.06% HX531, body weight decreased markedly during the first 4 days and by the end of the experiment was significantly lower than controls (Fig. 1A). In the animals given 0.03% HX531, the decrease in body weight became statistically significant on the 20th and 21st day of treatment.

In the animals given 0.06% HX531, food intake significantly decreased during the first 4 days and the level tends to be lower than controls through the experiment period, except for levels on the 10th, 12th, and 20th day of HX531 treatment (Fig. 1B). In the group administered 0.03% HX531, food intake level was lower than the controls, and the decrease in food intake became statistically significant during the first 2 days.

Rectal temperature was significantly higher in the first week of treatment in the 0.06% HX531 group compared with control animals (38.0°C ± 0.1°C vs 36.8°C ± 0.4°C, respectively). However, after the first week the temperature tended to decrease (Fig. 1C) and by the third week, the rectal temperature of the 0.06% HX531 group was lower than that of the control group (35.3°C ± 0.2°C vs 36.8°C ± 0.3°C, respectively). The rectal temperature of the 0.03% HX531 group was significantly higher than controls during the second week of treatment.

We used plasma 3-HB levels as an index of β-oxidation of free fatty acids (FFAs) (Fig. 1D). Compared with the control

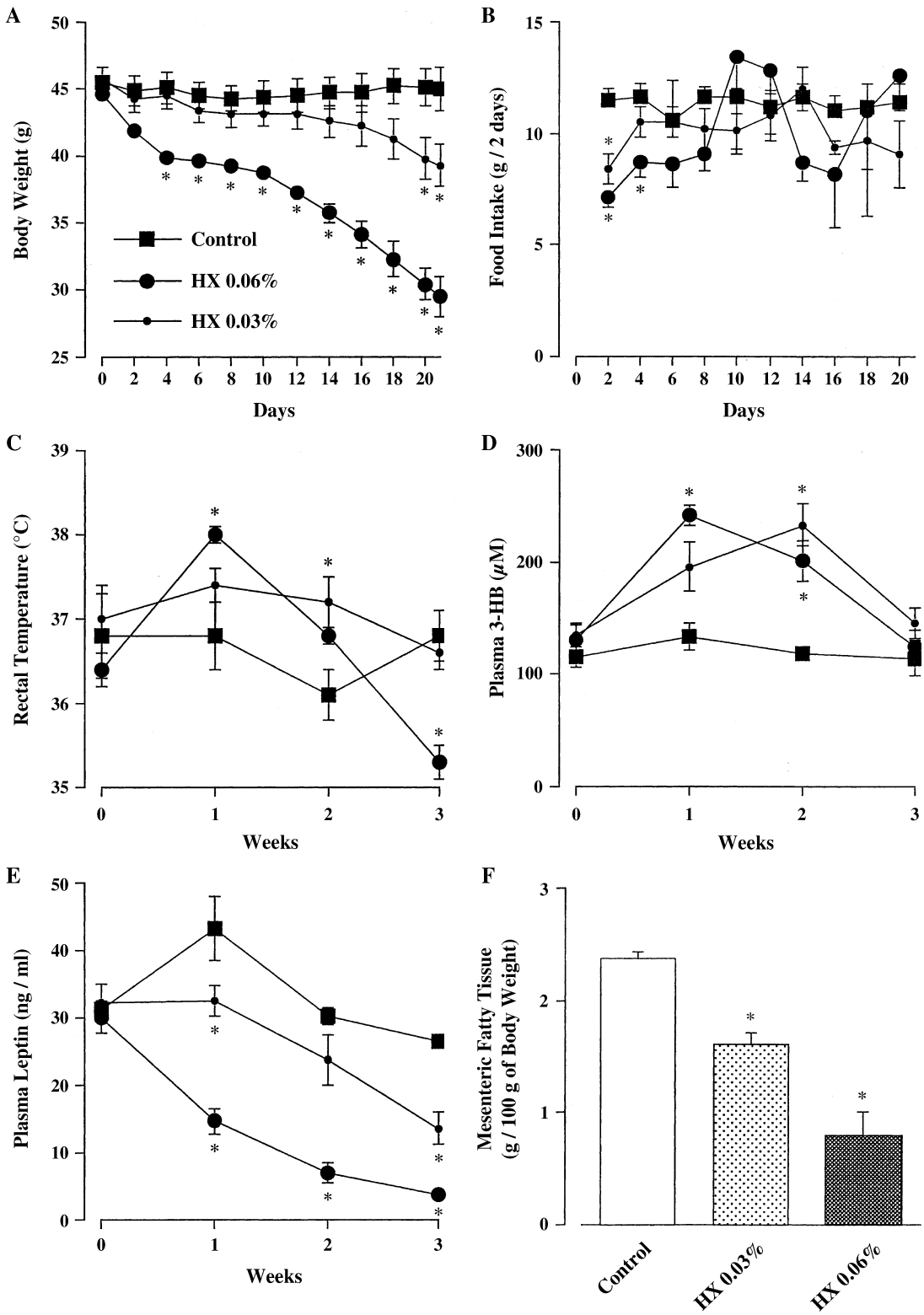
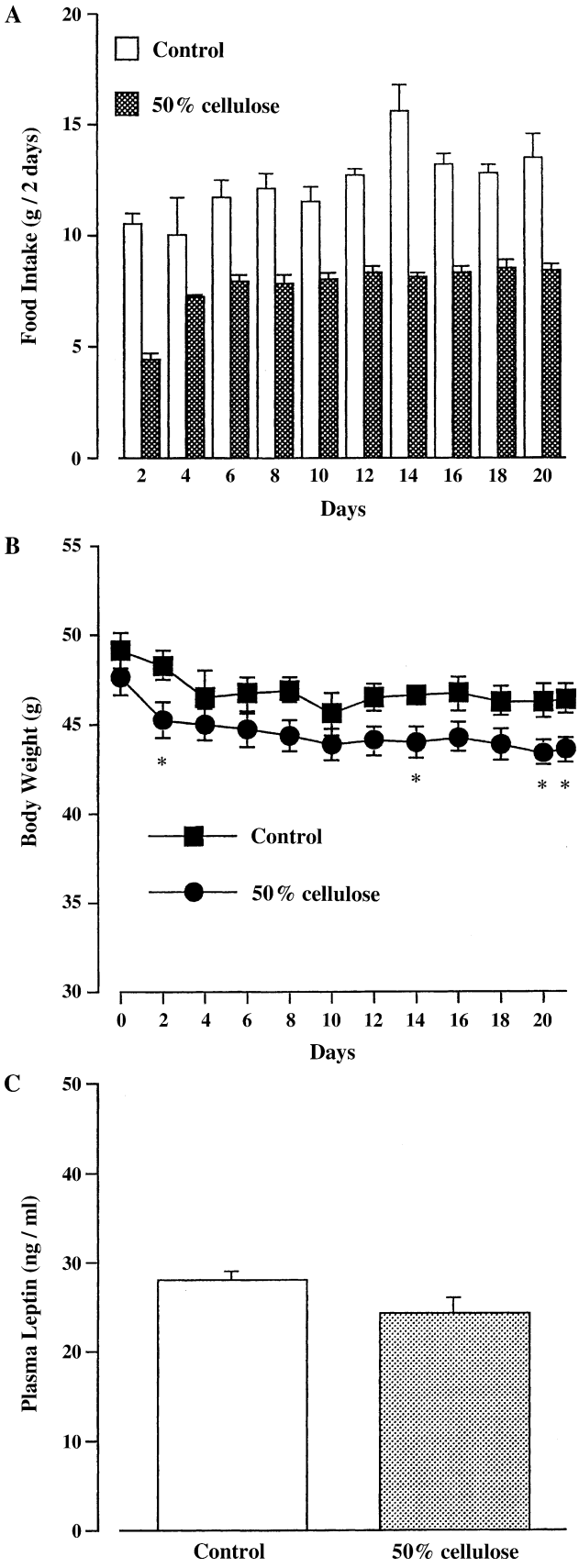


Fig. 1. Effects of HX531 in KK-A^y mice on (A) body weight, (B) food intake, (C) rectal temperature, (D) plasma 3-HB, (E) plasma leptin, and (F) weight of mesenteric fatty tissue. Values represent the mean ± SEM of 4 to 5 animals. Asterisk indicates $P < .05$, significantly different from controls (Dunnett multiple test).

group, HX531 (0.06%) increased 3-HB levels significantly in the first and second week of treatment in a dose-dependent manner. The 3-HB levels tended to decrease after reaching a peak in the first week. In contrast, in the 0.03% HX531 group, levels reached a peak in the second week before returning to basal levels in the third week (Fig. 1D). The time-related



changes in 3-HB levels associated with HX531 treatment followed similar trends to variations in rectal temperatures, as shown in Fig. 1C.

HX531 treatment for 3 weeks decreased plasma leptin levels significantly in a time- and dose-dependent manner (Fig. 1E). Within 1 week, both doses of HX531 decreased plasma leptin levels significantly (32.5 ± 2.3 and 14.7 ± 1.9 ng/mL in 0.03% and 0.06% HX531 groups, respectively), compared with controls (43.2 ± 4.8 ng/mL). By the third week, these levels had decreased to 13.6 ± 2.4 in the 0.03% HX531 group and 3.7 ± 1.1 ng/mL in the 0.06% HX531 group. This final level in the 0.06% HX531 group was almost the same as that measured in normal mice (ICR mice, n = 5, 2.6 ± 0.6 ng/mL; preliminary data).

Three weeks of treatment with HX531 also caused a dose-dependent decrease in the weight of mesenteric fatty tissue (Fig. 1F).

3.2. Effects of food intake restriction for 3 weeks on body weight and plasma leptin level in KK-A^y mice

To investigate whether food intake restriction affects body weight and plasma leptin level, we gave 50% cellulose food admixture to KK-A^y mice for 3 weeks. In the animals given 50% cellulose food admixture, food intake was restricted by 38%, compared with that of control group (Fig. 2A) (129 ± 5 and 80 ± 2 g/21 days in control and 50% cellulose group, respectively).

Food intake restriction significantly decreased in body weight on day 2, 14, 20, and 21; however, the effect throughout experiment period was weaker than that of 0.06% HX531 treatment shown in Fig. 1A (Fig. 2B).

After food intake restriction for 3 weeks, plasma leptin level in 50% cellulose group did not have significant difference from that of control group (Fig. 2C).

3.3. Effects of HX531 on leptin sensitivity in KK-A^y mice

Leptin sensitivity was assessed by reductions in food intake in response to exogenously administered leptin (5 μg/g body weight per day, IP). Our preliminary data showed that exogenously administered leptin (5 μg/g body weight per day, IP) for 4 days significantly decreased food intake in normal mice (ICR mice) (20.4 ± 1.1 g/4 days [n = 5] and 14.8 ± 1.2 g/4 days [n = 5] in control and leptin treatment group, respectively). On the other hand, food intake reduction in response to leptin in KK-A^y mice was not induced, indicating the animals were leptin resistant (23.0 ± 1.4 g/4 days [n = 5] and 23.9 ± 0.5 g/4 days [n = 4] in control and leptin treatment group, respectively). The administration at the dosage of leptin for 4 days did not significantly change

Fig. 2. Effects of food intake restriction on body weight (B) and plasma leptin level (C) in KK-A^y mice. In the animals given 50% cellulose food admixture, food intake level (grams per 2 days) was calculated as control powdered chow contents in the 50% cellulose admixture mice fed (A). Asterisk indicates $P < .05$, significantly different from control (Student t test).

body weight in both normal and KK-A^y mice, although that in normal mice tended to decrease.

Based on the result depicted in Fig. 1E, we choose 0.06% HX531 provided as a food admixture for 1 week as the dosage required to decrease plasma leptin levels. After this treatment regimen, leptin administered exogenously for 4 days decreased food intake significantly compared with controls (Fig. 3A). Throughout the experiment period, there was no significant difference between the 2 groups' body weights (Fig. 3B).

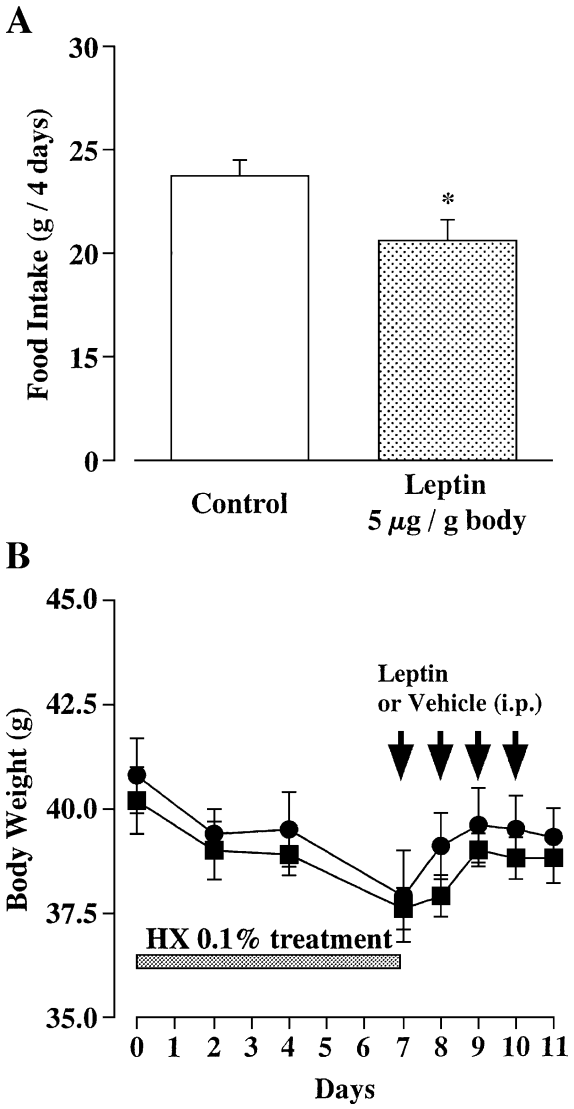


Fig. 3. Effects of HX531 on leptin sensitivity in KK-A^y mice. Leptin (5 μg/g body weight per day, n = 5) or vehicle (saline, n = 5) was administered to the mice for 4 days by IP injection after 1 week of HX531 treatment as a 0.06% food admixture. Through the period of leptin or vehicle administration, control powdered chow (CMF powder, Oriental Yeast Co Ltd) was provided to all the mice. A, Food intake during this 4-day period was measured to assess the effects of leptin administration. B, Change of body weight in mice throughout the experiment period. Circle and square symbols indicate leptin-administered group and vehicle-administered group, respectively. Values represent mean ± SEM. Asterisk indicates $P < .05$, significantly different from control (Student t test).

4. Discussion

The present study demonstrated that a minimum of 1 week of treatment with HX531, an RXR antagonist, improved leptin resistance in KK-A^y mice fed a normal diet. This finding is consistent with an earlier report by Yamauchi et al [17] that 1 week of HX531 treatment provided as a 0.1% admixture increased leptin sensitivity in KK-A^y mice fed a high-fat diet. Surprisingly, the improvement in leptin resistance we observed with HX531 was not associated by an increase in plasma leptin levels. This finding differs from that found in the study of Yamauchi and coworkers [17] and is the first report that HX531 may improve leptin resistance while decreasing plasma leptin levels.

Our data also supported the findings of this earlier study [17] that observed 2 weeks of HX531 treatment in KK-A^y mice decreased body weight and the weight of white adipose tissue [17]. In our study we measured mesenteric fatty tissue weight as an index of white adipose tissue. We also measured rectal temperature and plasma 3-HB as an index of energy expenditure and β-oxidation of FFA, respectively. Our data showed that HX531 caused a dose-dependent increase in plasma 3-HB levels that subsequently returned gradually to basal levels. The time elapsed before peak plasma 3-HB levels were reached tended to be delayed with decreasing dosages of HX531. The pattern of changes in plasma 3-HB levels was also observed to be similar to the pattern of changes in rectal temperature measurements. In the earlier study on KK-A^y mice fed a high-fat diet, it was reported that HX531 treatment increased expression of enzymes involved in β-oxidation, such as peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, acyl-CoA oxidase, and uncoupling protein 2 [17]. It therefore appears likely that HX531 initially decreases adipose tissue content in the body, with the FFAs released from the adipose tissue then undergoing β-oxidation in various peripheral tissues such as the liver. Herein, we also showed that food restriction did not decrease body weight in KK-A^y mice as strong as 0.06% HX531 admixture treatment, and plasma leptin was not decreased. These results strongly suggest that (1) HX531 does not decrease in body weight account for food intake reduction, (2) but, HX531 has an effect to decrease in adipose tissue, and (3) decrease in plasma leptin level with HX531 treatment may due to decrease in adipose tissue in the body.

As it is well known that leptin decreases food intake and body weight [1-3], our finding of elevated plasma leptin levels in the KK-A^y mice suggests these animals are "leptin resistant" [10,18]. In the present study, treatment for 1 week with HX531 as a 0.06% food admixture resulted in a decrease in plasma leptin levels in these animals (Fig. 1E) associated with a return to normal leptin reactivity (Fig. 3A). In the earlier study in these mice it was found that although HX531 treatment improved leptin resistance, this effect was accompanied by an increase in plasma leptin levels [17]. These

results were interpreted as indicating that RXR antagonism with HX531 increased both plasma levels and sensitivity of leptin. The difference between the 2 studies may be due to variations in diet during the treatment periods, as in our study, HX531 treatment was introduced under normal dietary conditions, whereas in the study of Yamauchi et al treatment was administered with fatty food [17]. Under normal food conditions, HX531 decreased body weight and mesenteric fatty tissue weight in a dose-dependent manner, in contrast to a high-fat diet and HX531 treatment that resulted in an increase in body weight. Both of these findings are consistent with evidence that plasma leptin levels and adipose tissue mass are positively correlated [5]. Taken together these results indicate HX531 may improve leptin resistance at the level of the receptor and/or subsequent signaling pathways, although the exact mechanism of these actions remains unclear. Although the effect of HX531 appears to be independent of plasma leptin level, our results demonstrate clearly that HX531 enhances the effects of leptin by improving sensitivity rather than increasing levels.

Exogenously administered leptin (5 μg/g body weight per day, IP) for 4 days did not decrease body weight in KK-A^y mice (Fig. 3B), although food intake was decreased in response to the leptin (Fig. 3A). Our preliminary study using normal mice (ICR mice) showed that exogenously administered leptin (5 μg/g body weight per day, IP) on the same administration schedule tended to decrease body weight of the mice; however, the decreasing level was not significant. These results suggest that the dosage of leptin (5 μg/g body weight per day, IP) and/or the treatment period (4 days) may not be enough to decrease body weight in mice.

In conclusion, an RXR antagonist, HX531, was administered for 3 weeks as a food admixture to KK-A^y mice fed a normal diet. This treatment resulted in a decrease in body weight and mesenteric fatty tissue weight in the animals. Initially, HX531 also caused a dose-dependent rise in plasma 3-HB levels and rectal temperature, with these changes then reducing gradually to normal levels. These results suggest that HX531 firstly decreases the fatty tissue content in the body, with the FFA released from this tissue then undergoing β-oxidation in peripheral tissues. HX531 treatment for 3 weeks in KK-A^y mice under normal food condition decreased plasma leptin levels in a dose- and time-dependent manner. After 1 week of HX531 treatment as a 0.06% food admixture, leptin levels had decreased in these animals whereas leptin resistance was attenuated. These results suggest that HX531 decreases plasma leptin levels accompanied simultaneously by a decrease in fatty tissue content in the body and an improvement in leptin resistance.

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