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Metabolism Clinical and Experimental 60 (2011) 327–334
Metabolism
Clinical and Experimental
www.metabolismjournal.com

The anorexigenic effects of metformin involve increases in hypothalamic leptin receptor expression

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Received 8 October 2009; accepted 8 February 2010

Abstract

Metformin demonstrates anorectic effects in vivo and inhibits neuropeptide Y expression in cultured hypothalamic neurons. Here we investigated the mechanisms implicated in the modulation of feeding by metformin in animals rendered obese by long-term high-fat diet (diet-induced obesity [DIO]) and in animals resistant to obesity (diet resistant [DR]). Male Long-Evans rats were kept on normal chow feeding (controls) or on high-fat diet (DIO, DR) for 6 months. Afterward, rats were treated 14 days with metformin (75 mg/kg) or isotonic sodium chloride solution and killed. Energy efficiency, metabolic parameters, and gene expression were analyzed at the end of the high-fat diet period and after 14 days of metformin treatment. At the end of the high-fat diet period, despite higher leptin levels, DIO rats had higher levels of hypothalamic neuropeptide Y expression than DR or control rats, suggesting a central leptin resistance. In DIO but also in DR rats, metformin treatment induced significant reductions of food intake accompanied by decreases in body weight. Interestingly, the weight loss achieved by metformin was correlated with pretreatment plasma leptin levels. This effect was paralleled by a stimulation of the expression of the leptin receptor gene (ObRb) in the arcuate nucleus. These data identify the hypothalamic ObRb as a gene modulated after metformin treatment and suggest that the anorectic effects of the drug are potentially mediated via an increase in the central sensitivity to leptin. Thus, they provide a rationale for novel therapeutic approaches associating leptin and metformin in the treatment of obesity.

1. Introduction

Obesity results from an imbalance between food intake and energy expenditure. It is currently hypothesized that in any given individual, the net food intake results from an equilibrium between the activity of neurons promoting (orexigenic) and neurons inhibiting (anorexigenic) feeding [1]. These specialized neurons are mainly located in hypothalamic nuclei and areas, as well as in hindbrain areas such as the nucleus of the solitary tract [2]. The equilibrium between orexigenic and anorexigenic outputs arises from the integration of humoral as well as neuronal peripheral signals and is key to achieving the tight regulation of feeding and energy expenditure observed over time in most species [3]. Although there has been a recent explosion in knowledge about neural regulatory systems involved in energy balance, the epidemic of obesity on an unchanging genetic background suggests powerful environmental forces that dysregulate energy balance. Long-term access to a high-energy diet is certainly one external factor likely to be a major component [4].

Given the complexity and redundancy of this system, it may appear useful in the design of future drug therapies of obesity to associate different agents modulating parallel (and potentially antagonist) feeding pathways in a synergistic manner. In this respect, metformin appears as an interesting candidate for such an association. Metformin is a widely used oral glucose-lowering agent that improves insulin sensitivity in peripheral target organs like skeletal muscle, fat tissue, and liver [5]. In addition, metformin has long been suggested clinically to reduce food intake in diabetic and nondiabetic patients [6-8].

Recent data suggest that the potential anorexigenic action of metformin results from specific effects at the level of the

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0026-0495/$ – see front matter © 2011 Published by Elsevier Inc.
doi:10.1016/j.metabol.2010.02.007

hypothalamic centers regulating satiety and feeding [9,10]. In diet-induced obese rats, Kim et al [9] show that metformin enhances the hypothalamic phosphorylation of STAT3 induced by acute intracerebroventricular leptin injections. In primary hypothalamic neuronal cell cultures, we observed that metformin was able to block entirely the phosphorylation of adenosine monophosphate–activated protein kinase (AMPK) normally observed in low glucose conditions [10]. As a result, the rise in neuropeptide Y (NPY) that follows a lowering of glucose levels in the culture medium of hypothalamic neurons was also completely prevented by metformin in that system [10]. The latter observation could be relevant to previous studies suggesting that metformin may decrease food intake because AMPK has been implicated in the appetite-modulating effects of different circulating factors at the level of the central nervous system [11,12] and NPY remains one of the most potent orexigenic neuropeptides known to date [13]. Taken together, our observation [10] and data from Kim et al [9] suggest that metformin, which acts in peripheral tissues by activating AMPK [14], may possibly operate via different mechanisms at the level of the central nervous system. This hypothesis is also supported by previous observations demonstrating that orally administered metformin can cross the blood-brain barrier [15].

Here we evaluated the potential of metformin administered peripherally to modulate food intake and hypothalamic gene expression in rodents fed a high-fat diet for 6 months. Using this model, we demonstrate that a subset of animals is completely resistant to the obesity-inducing effects of high-fat feeding. We could also demonstrate that 2 weeks of metformin administration to rats fed high-fat diet for 6 months induced significant weight losses in all animals, an effect that was completely independent of the phenotype (whether obese or resistant). This was in sharp contrast with metformin administration to control rats fed a normal chow, in which the drug had no effect on body weight. Weight loss in high-fat–fed animals was accompanied with significant decreases in total calorie intake during metformin administration and with an increase in ObRb expression in the hypothalamic arcuate nucleus. The latter observation resonates with the fact that metformin-induced weight loss was directly correlated with pretreatment leptin levels in these animals. Taken together, these data demonstrate that metformin is affecting the central nervous system control of food intake and that a modulation of the equilibrium of leptin-leptin receptor system is directly or indirectly implicated.

2. Material and methods

2.1. Study design

All studies were formally approved by the State Ethical Committee on animal experimentation.

2.2. High-fat feeding

Because of our previous results in neuronal cell cultures suggesting that metformin may be modulating hypothalamic NPY expression [10], we choose to test the effects of metformin in an in vivo model of high NPY expression correlated obesity. To this end, we conducted a preliminary experiment designed to evaluate hypothalamic NPY gene expression after 2 months of high-fat feeding. Twenty-six male Long-Evans rats were purchased immediately after weaning (Elevage Janvier, Genest-St Isle, France). Upon arrival at our laboratory, they were housed individually under a 12-hour light/12-hour dark cycle with food and water ad libitum. Animals were handled every day by the same person; and after 1 week of adaptation to their new environment, they were divided into 2 groups : controls (CT, n = 6), which were fed a normal chow (Kliba Nafag 3200, Provimi Kliba AG, Kaiseraugst, Switzerland), and high-fat–fed animals (n = 20), which were put for 2 months on a commercially available high-fat chow containing 4.80 kcal/g with 24% carbohydrate, 21% protein, and 55% fat (TD93075, Harlan Teklad, Madison, WI). Food intake and body weights were measured daily for 2 months, after which animals were killed by decapitation between 8:00 and 10:00 AM. Hypothalami were quickly dissected out and flash-frozen in liquid nitrogen. Because of the variability observed in the weight gain of animals in the high-fat–feeding group, the 5 animals with the highest body weight at 6 months constituted the diet-induced obese group (DIO); and the 5 rats with the lowest body weight constituted the diet-resistant group (DR).

Because the levels of hypothalamic gene expression were not different from controls after 2 months of high-fat diet, high-fat feeding was extended to 6 consecutive months for the subsequent experiment. To this end, 60 male Long-Evans rats were purchased from Janvier immediately after weaning, housed, and handled as described above for the first week. Ten of these rats (CT) were kept long term on normal chow feeding (Kliba Nafag 3200), whereas the others (n = 50) were put for 6 months on the high-fat diet described above. Animals were fed ad libitum throughout the experiment: fresh food was added when needed in controlled amounts, and body weights were measured weekly. At the end of the high-fat feeding period, energy efficiency was calculated by dividing the individual body weight gain over 6 months by the total caloric intake over the same period.

After 6 months, we confirm the previously reported variability in body weight gain of animals fed a high-fat diet [16], with a significant number of rats displaying no excess weight compared with controls. Therefore, the 10 animals with the lowest weight were assigned to a group called *DR*, whereas the 10 animals with the highest body weight were assigned to a group called *DIO*. The remaining animals (n = 30) were discarded from the experiment, and all further analyses were performed using animals in these 3 groups (DIO, DR, and CT).

2.3. Metformin treatment

Before starting metformin administration, a blood sample was obtained from the jugular vein of all rats in the CT, DR, and DIO groups under light halothane anesthesia. This allowed us to measure baseline insulin, leptin, and glucose levels. Animals were then randomized and weight matched within each group to either metformin or placebo treatment (n = 5 in each subgroup) for an additional 2 weeks, during which they were kept on the same diet as during the preceding 6 months. Metformin (75 mg/kg) was administered daily, between 4:00 and 6:00 PM, via intraperitoneal injections, whereas animals in the placebo groups received the same volume of intraperitoneal isotonic sodium chloride solution injections. Body weights and food intake were measured daily during this 2-week period, at the end of which animals were killed by decapitation between 8:00 and 10:00 AM. Trunk blood was collected in 10 mmol/L EDTA and immediately centrifuged, and the plasma was kept frozen at −20°C until use.

Leptin and insulin were measured by radioimmunoassay using commercially available kits (Linco Research, St Charles, MO). The intra- and interassay coefficients of variability for leptin were as follows: 11.2% at 0.4 ng/mL and 4.9% at 5.4 ng/mL (intra); 14.6% at 0.4 ng/mL and 3.3% at 5.4 ng/mL (inter). For insulin, they were: 2.2% at 0.5 ng/mL and 4.6% at 3.7 ng/mL (intra); 8.9% at 0.5 ng/mL and 9.4% at 3.7 ng/mL (inter). Blood glucose levels were determined with the Glucometer Elite XL (Bayer, Zurich, Switzerland).

2.4. Hypothalamic dissection

Whole brain was dissected out and flash-frozen in liquid nitrogen. Frozen brains were then processed by cutting serial 1-mm–thick sections at −14°C, using a microtome (Model HM430; Microm International, Walldorf, Germany). One sagittal cut was made to bisect the brain, followed by 2 cuts left and right of the bisectional cut to produce 1-mm–thick sagittal section left and right of the third ventricle. Landmarks depicted in the atlas of Swanson as a guide like fornix, optic tracts, and mammillary nuclei were used to dissect reproducible piece of arcuate nucleus. The 2 tissue pieces were combined and stored at −80°C until further use [17].

2.5. Gene expression measurements

Total RNA was extracted from tissue samples using a commercially available kit (RNeasy; Qiagen, Hombrechtikon, Switzerland) and following the manufacturer's instructions. First-strand complementary DNA was synthesized from 1 μg of total RNA in a 20-μL reaction volume using random primers (Promega, Wallisellen, Switzerland) and Superscript II (Invitrogen, Carlsbad, CA).

Relative messenger RNA (mRNA) levels of NPY, proopiomelanocortin (POMC), Agouti gene–related peptide (AgRP), and β2-microglobulin (used as endogenous control) were then assessed by real-time quantitative polymerase chain reaction using the LightCycler technology (Roche Diagnostics, Rotkreuz, Switzerland) with Takara SYBR Green (Takara Bio, Shiga, Japan). Complementary DNAs of interest were amplified using the following specific primers (Microsynth, Windish, Switzerland): sNPY (5′-tccgctctgcgacactacat-3′) and asNPY (5′-tgctttctctcattaagagatctga-3′); sAgRP (5′-cgtgtgggccctttattaga-3′) and asAgRP (5′-agtacctagcttgcggcagt-3′); sPOMC (5′-gaaggtgtaccccaatgtcg-3′) and asPOMC (5′-cttctcggaggtcatgaagc-3′); sObRb (5′-gcaggttccagcttcttgag-3′) and asObRb (5′-tgacagcttgatgccaacat-3′); sβ2-microglobulin (5′-gagcccaaaaccgtcacc-3′) and asβ2-microglobulin (5′-gaagatggtgtgctcatt-3′). All samples were quantified in at least 2 runs, and a negative control reaction in the absence of template was always added for each primer pair. Interassay coefficients of variation were always less than 10%. Relative expression was then determined using crossing point values and amplification efficiencies of the target gene and the reference gene.

2.6. Data analysis

All data are expressed as means ± SEM, and statistical significance was assessed by 2-way analysis of variance with Bonferroni post hoc corrections as well as nonparametric Kruskal-Wallis test. Overall effects of metformin were assessed by group adjusted means between metformin and placebo. $P < .05$ was considered a significant difference. Correlations were assessed by Spearman nonparametric correlation test.

3. Results

3.1. Effects of 6 months of high-fat diet

Fig. 1 summarizes the evolution of body weights and food intake during 6 months of normal chow (CT) or high-fat feeding (DR and DIO). Diet-induced obese rats exhibited a significant increase in body weight compared with both the CT rats and the DR rats. This difference became significant at 4 weeks of treatment and persisted throughout the entire experiment (Fig. 1A). After 6 months, but before metformin administration, mean body weights were 505.4 ± 15.9 g for CT rats, 516.0 ± 6.7 g for DR (not significant), and 613.7 ± 9.5 g for DIO animals ($P < .01$ vs CT and DR, Fig. 1B).

Fig. 1C illustrates the evolution of cumulated food intake in the 3 groups over 6 months. Food intake became significantly different between DR and DIO rats as early as after 2 weeks of high-fat diet (intake higher in DIO), a difference that persisted throughout the experiment. Food intake in the DIO group was also higher than that in controls, a difference that became significant after 4 weeks. On average over 6 months, animals in the DIO group ingested spontaneously 7.3% more calories

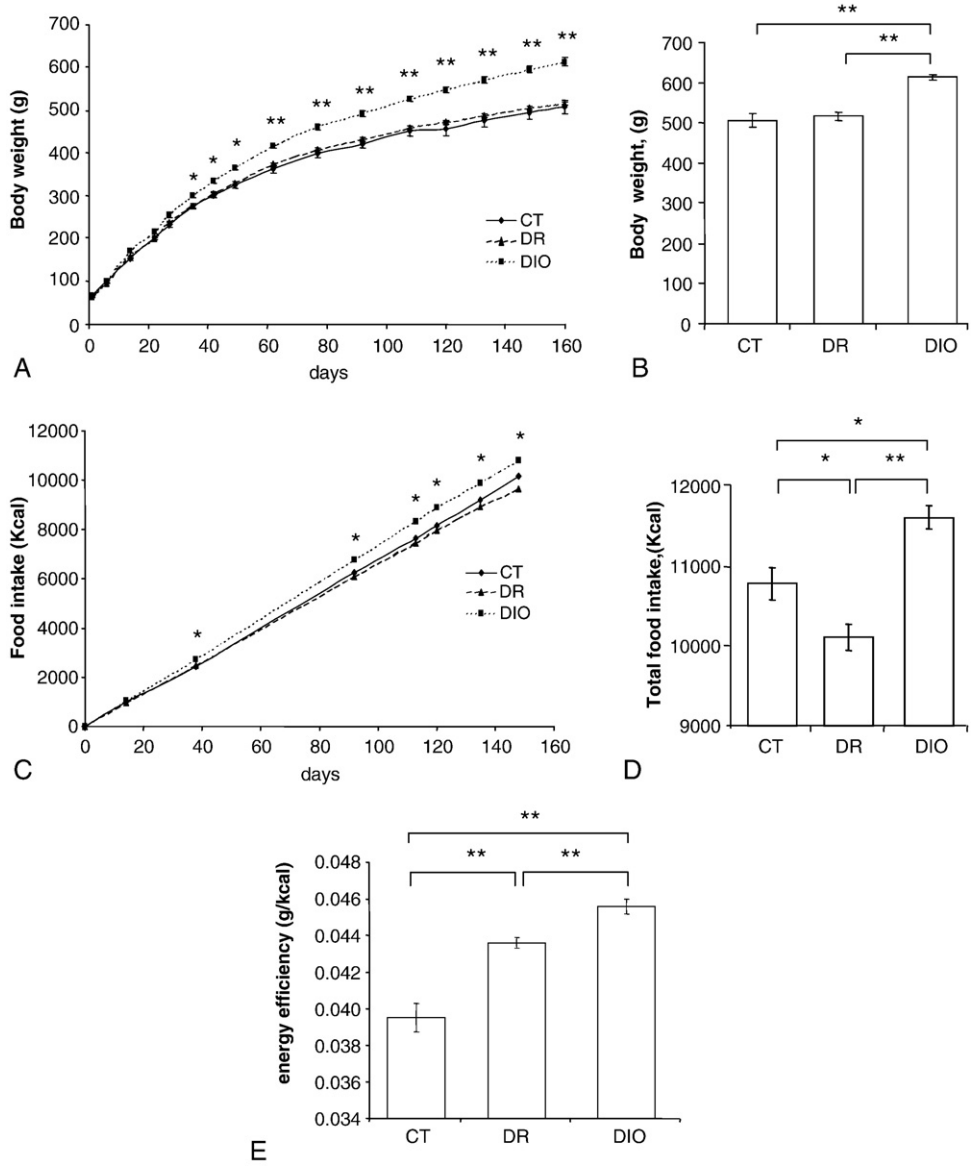

Fig. 1. Evolution of body weights (A) and food intake (C) during 6 months of controlled diet. Total body weights (B) and cumulative total food intake (D) at the end of the controlled diet. The calculated energy efficiency over 6 months (E) in the 3 groups of animals. Data are means ± SEM of 10 rats per group. *P <. 05, **P < .01.

than CT and 15.2% more than DR animals (11671 ± 165 kcal for DIO vs 10823 ± 208 kcal for CT [P < .05] and 10135 ± 145 kcal for DR [P < .01], Fig. 1D). The lowest food intake was observed in animals from the DR group (6.36% less than CT, P < .05).

Fig. 1E displays the mean energy efficiency of the food administered, calculated within each group over 6 months. There were significant differences between each group, the highest efficiency being observed in DIO and the lowest in CT animals.

Fig. 2 displays the levels of insulin and leptin after 6 months of normal or high-fat diet. Insulin was significantly higher in DIO than in CT rats, whereas it was comparable to CT in DR animals (13.6 ± 1.3 ng/mL in DIO vs 5.0 ± 0.6 ng/mL in DR and 4.8 ± 0.7 ng/mL in CT, P < .01 for both). In contrast, leptin was increased in both DIO and DR rats compared with CT (21.58 ± 1.4 ng/mL in DR and 32.52 ± 7.49 ng/mL in DIO vs 16.13 ± 1.13 ng/mL in CT, P < .05 for both).

Analysis of hypothalamic arcuate nucleus NPY, AgRP, or POMC expression levels after 2 months of controlled diet disclosed no difference between normal chow and high-fat–fed animals (preliminary experiment, data not shown). Fig. 3 displays the levels of expression of these genes in the arcuate nucleus of rats after 6 months of controlled diet. These data correspond to the animals that received 14 days of intraperitoneal saline injections after 6 months of controlled diet (n = 5 in each group). They

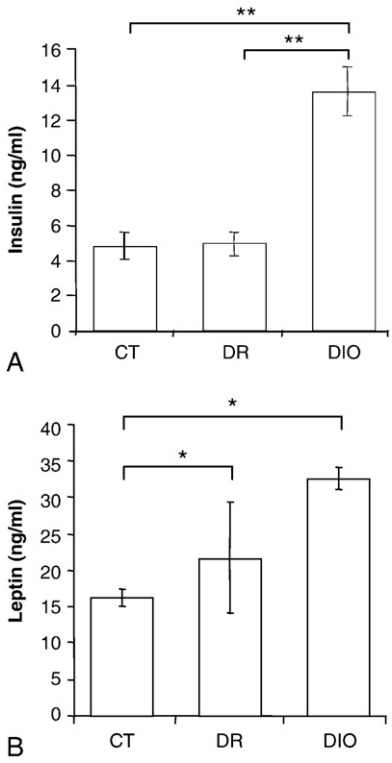

Fig. 2. Plasma insulin (A) and leptin (B) levels at the end of the controlled feeding period in the 3 groups of animals. Data are means ± SEM of 10 rats per group. *$P < .05$, **$P < .01$.

illustrate the increases observed for the 3 genes in rats from the DIO group compared with either DR or CT (for NPY and POMC, $P < .05$ compared with CT and DR).

3.2. Effects of metformin

Fig. 4 illustrates the effects of 14 days of metformin or saline administration in the 3 groups of animals. As demonstrated by data displayed in Fig. 4A, we observed a significant decrease in insulin levels in the animals receiving metformin. In contrast, leptin levels were not affected by the drug (data not shown).

The daily changes in body weights in the 3 groups of animals during 14 days of daily intraperitoneal metformin administration (75 mg/kg) are illustrated in Fig. 4B-D. In CT animals, we did not observe any modulation of body weight by the drug (Fig. 4B). In contrast, it was found to decrease significantly the body weights of animals both in the DR (−1.2 ± 3.2 g vs −11.0 ± 1.8 g for saline and metformin, respectively; $P < .05$) and in the DIO (+1.2 ± 2.0 g vs −13.2 ± 4.0 g for saline and metformin, respectively; $P < .05$) groups (Figs. 4C,D). This effect on the evolution of body weight was at least in part due to a decrease in food intake, as demonstrated by the significantly lower total calorie intake over 14 days observed in DR and DIO animals receiving metformin compared with rats treated with saline injections (657.6 ± 6.4 for DR + metformin vs 742.1 ± 29.5 kcal for DR + saline; 746.9 ± 30.6 kcal for DIO + metformin vs 845.8 ± 34.8 for DIO + saline; $P < .05$ for both; Fig. 2D).

Contrasting with these effects of metformin on body weight and food intake, we found no difference in the hypothalamic expression of NPY, AgRP, or POMC between saline-treated and metformin-treated animals in any of the 3 groups (data not shown).

3.3. Metformin and the leptin system

Fig. 5A represents the correlation between pretreatment leptin levels and the change in body weight measured after metformin administration, with animals receiving metformin from all 3 groups included in the analysis. These data demonstrate that the change in body weight achieved by 14 days of metformin administration was significantly correlated with pretreatment plasma leptin levels: weight loss was greater in animals with the highest plasma leptin levels. This observation prompted us to evaluate the levels of expression of the leptin receptor in the hypothalamic arcuate nucleus of rats in these 3 groups of animals. Fig. 5B demonstrates that metformin was found to increase the levels of ObRb mRNA in all treated animals (Fig. 5D).

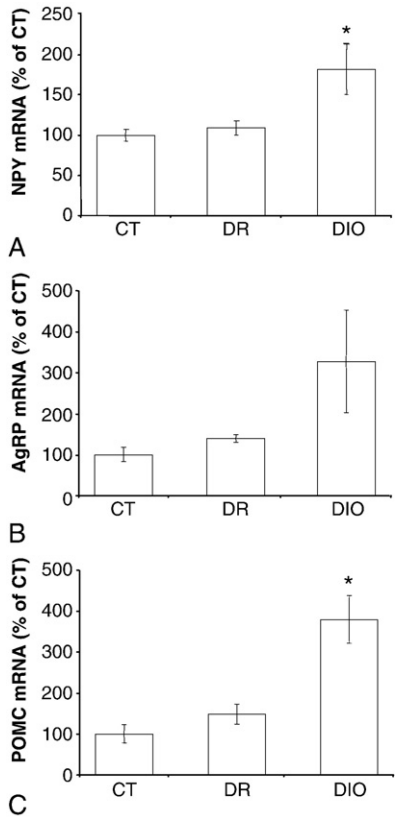

Fig. 3. Neuropeptide Y (A), AgRP (B), and POMC (C) mRNA levels in hypothalamic arcuate nucleus extracts from placebo-treated (saline) animals in the 3 groups. Data are means ± SEM of 5 rats per group. *$P < .05$ vs CT or DR.

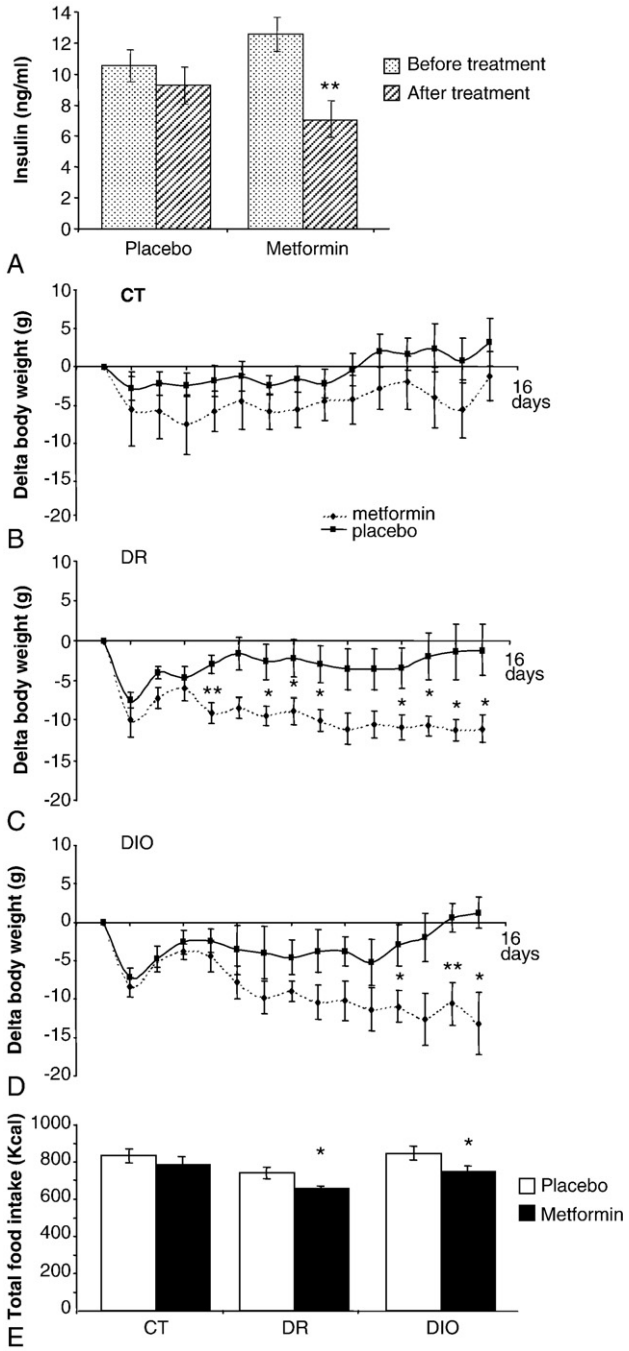

Fig. 4. A, Plasma insulin levels in animals receiving 14 days of treatment with either placebo (saline) or metformin, measured immediately before the start of treatment and at sacrifice. Daily evolution of body weights during 14 days of treatment with either placebo or metformin in animals from the control group (B), in diet-resistant animals (C), and in diet-induced obese animals (D). E, Cumulative food intake over 14 days in placebo-treated (□) and metformin-treated (■) animals from the 3 groups. *$P < .05$, **$P < .01$ vs placebo.

Interestingly, study of ObRb expression in fat and liver tissues of the same rats showed no modification of the receptor mRNA level (data not shown), suggesting a central effect of the drug.

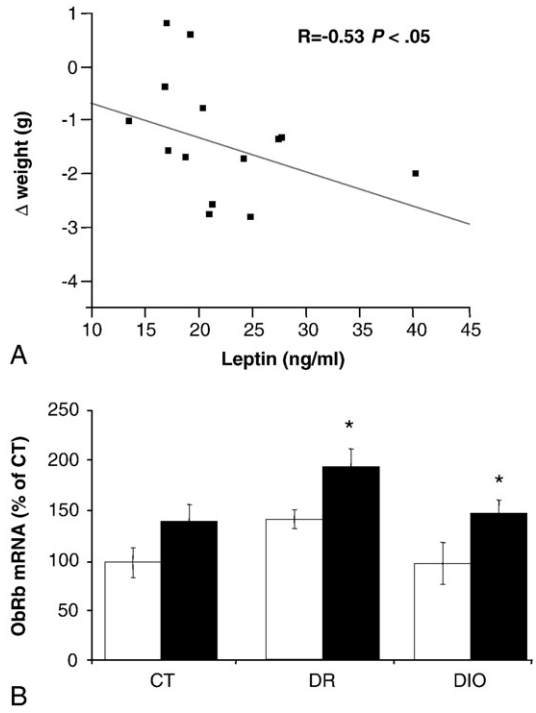

Fig. 5. A, Correlation between pretreatment plasma leptin levels and Δ body weight achieved after 14 days of metformin administration. This graph includes all animals receiving metformin regardless of their group (CT, DR, or DIO). B, ObRb mRNA levels in extracts from the hypothalamic arcuate nucleus after 14 days of metformin (■) or placebo (□) treatment in animals from the 3 groups. Data are means ± SEM of 5 rats per group *$P < .05$ vs placebo.

4. Discussion

Given the alarming pace of the development of the obesity epidemics worldwide, there is an urgent need for novel therapeutic approaches. In this perspective and given the relative lack of available long-term data, a better understanding of the molecular mechanisms governing feeding and metabolic adaptations by the central nervous system appears mandatory. The primary aim of our study was to investigate the potential central mechanisms of action of metformin in the modulation of feeding. To this end, we used 3 different groups of animals: DIO, DR, and CT.

Here we found that after 2 months of high-fat or control diet, no modification in the expression of key central peptide modulators of feeding could be observed despite clear phenotypic differences between animals in the 3 groups (data not shown). In contrast, NPY, AgRP, and POMC expression levels were all modified in the arcuate hypothalamic nucleus of rats rendered obese by 6 months of high-fat diet. As expected [18], the level of expression of NPY and AgRP was higher in DIO rats than in animals from either the DR or the CT groups. In these 2 latter groups, the level of expression of the 2 neuropeptides was similar. We also observed an increase in POMC mRNA levels in DIO rats compared with animals in the DR or CT groups. Overall, these changes

occurring in the face of high leptin levels in DIO rats are consistent with central leptin resistance at the level of NPY-expressing neurons, whereas POMC-expressing neurons seem to retain their physiologic regulation [19]. Such hypothesis would be consistent with previous data demonstrating site-specific resistance to leptin within the hypothalamus [17].

In addition to providing the first set of data on hypothalamic gene expression levels in long-term high-fat diet, these results illustrate the time course of apparition of these changes. Indeed, we found no difference in gene expression at 2 months in animals of identical genetic background submitted to the exact same protocol. This observation resonates with previous data indicating that, over the course of development of high-fat–induced obesity, the condition is first reversible and then becomes irreversible [20]. This evolution has been attributed to permanent changes in neural connections [21], and our data now suggest that changes in gene expression levels may also participate to this evolution.

To better delineate the potential feeding effects of metformin, the drug was administered not only to high-fat obese animals, but also to animals resistant to high-fat–induced obesity. As expected, we found that metformin can induce significant weight losses in high-fat–fed obese rats. This effect was at least partially mediated via a modulation of food intake, as demonstrated by the observation of a concomitant decrease in the total caloric intake of metformin-treated animals compared with the placebo groups. Therefore, our data are entirely consistent with the relatively scarce literature suggesting that metformin is exerting central anorexigenic effects [22,23]. Surprisingly, we also observed significant weight losses in rats resistant to the obesity-inducing effects of high-fat diet, despite their similarity to controls with respect to both body weight and hypothalamic NPY, AgRP, and POMC expression levels.

Blood leptin level before treatment was the only parameter that differed between the DR and CT groups in our study, prompting us to evaluate the correlation between weight loss and plasma leptin levels at the beginning of the treatment. The results demonstrate that the anorexigenic action of metformin was dependent upon pretreatment circulating leptin levels, suggesting a role for the leptin system in this effect of metformin. We could then demonstrate that metformin increases the levels of expression of ObRb in hypothalamic neurons of the arcuate nucleus. Interestingly, we did not observe any modulation of ObRb receptor expression levels in peripheral tissues, thus suggesting the specificity of this effect of metformin for the central nervous system. This observation, coupled with the correlation existing between circulating leptin and the degree of weight loss achieved, suggests that metformin may potentially increase the sensitivity of hypothalamic neurons to leptin. In addition, our data also indicate that raising hypothalamic expression of ObRb will eventually modulate overall feeding regulations by the central nervous system only in situations of high endogenous leptin tone. A similar reasoning may also provide a potential explanation to some of the inconsistencies of the current literature regarding the effects of metformin on feeding, both in rodents and in humans [24]. Finally, it also raises the possibility that circulating leptin levels may be a marker of sensitivity to the anorexigenic effects of metformin.

Previous data have shown that the induction of STAT3 phosphorylation by leptin in hypothalamic neurons is stronger in the presence of metformin [9]. By providing a potential mechanism for this effect, our data now expand upon these previous results in 2 important ways. First, we could identify arcuate nucleus ObRb as a potential gene regulated by the drug in the central nervous system. It is not entirely clear at present whether this modulation results from a direct effect of metformin upon hypothalamic neurons, or rather from an indirect action via one or several unidentified humoral or neuronal mediators. However and regardless of the mechanism implicated, our data demonstrate that, when administered peripherally, metformin results in changes in ObRb expression levels in the hypothalamus but not in the liver or adipose tissue. Further experiments will be necessary to clearly identify the molecular and cellular mechanisms leading to ObRb induction in hypothalamic neurons. Second, we observed these effects of metformin using a fourth of the dose administered previously [9]. Because the amount that we used here is very close to the doses of metformin used in humans characterized by long-term obesity [25], our data are likely to be relevant for human physiopathology.

In conclusion, the present data suggest that human obesity should constitute a particularly good indication for a drug that ameliorates the sensitivity of the central nervous system to leptin because this condition is characterized by leptin resistance [26]. The identification of the leptin receptor as a potential target of metformin action in the central nervous system, together with our divergent observations in lean and obese models, is providing the proof of principle in favor of a combination treatment associating metformin to leptin in obesity. The rationale for such association can be directly derived from diabetes treatment using insulin in combination with metformin [27]. Further work needs to be performed to delineate the precise mechanism by which metformin can alter leptin receptor expression.

Acknowledgment

The authors wish to thank Luc Tappy, Marta Korbonitz, Blerina Kola, and Vittorio Giusti for helpful discussions.

This work was supported by grants from the Swiss National Science Foundation (320000-112075 and 310000-122094) to FPP.

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