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Possible common central pathway for resistin and insulin in regulating food intake

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Abstract

Aim: Adipose tissue has been the object of intense research in the field of obesity and diabetes diseases in the last decade. Examination of adipocyte-secreted peptides led to the identification of a unique polypeptide, resistin (RSTN), which has been suggested as a link between obesity and diabetes. RSTN plays a clearly documented role in blocking insulin (INS)-induced hypoglycaemia. As brain injection of INS affects feeding behaviour, we studied the possible interaction between INS and RSTN in food-deprived rats, measuring effects on food intake. In addition, we examined how RSTN might affect neuropeptide Y (NPY)-induced feeding, as studies have shown that rat RSTN can interfere with the NPY system.

Methods: Overnight food-deprived rats were injected into the third brain ventricle (3V) with either INS (10 or 20 mUI), RSTN (0.1–0.4 nmol/rat), or saline before access to food. Another group of rats was injected into the 3V with RSTN alone, NPY alone or RSTN plus NPY. Their food intake and body weight were measured.

Results: Our results confirm the hypophagic effect of RSTN on food deprivation-induced food intake, and more importantly, show that RSTN neither potentiates nor blocks the effects of INS on food intake, but does reduce the hyperphagic effect of NPY.

Conclusion: The observation that RSTN does not modify feeding INS-induced hypophagia, but does influence NPY-induced feeding, points to the possibility that RSTN may be involved in control of food intake through an NPY-ergic mechanism as INS.

Keywords adipocyte-derived peptides, food intake, insulin, neuropeptide Y, resistin.

Adipose tissue has been the object of intense research in the field of obesity and diabetes diseases in the last decade, with considerable attention devoted to its products, such as leptin and adiponectin. Examination of adipocyte-secreted peptides led to the identification of a unique polypeptide, resistin (RSTN) (Holcomb *et al.* 2000, Kim *et al.* 2001, Steppan *et al.* 2001), which has been suggested as a link between obesity and diabetes (Steppan *et al.* 2001). In particular, it has been shown that RSTN impairs glucose tolerance and insulin (INS) activity and that its neutralization enhances INS-stimulated glucose uptake in adipocytes (Steppan *et al.* 2001). A possible role of RSTN in the regulation of food intake and body weight is suggested by the high

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serum concentrations of RSTN in ob/ob and db/db mice as well as in diet-induced obese mice (Steppan *et al.* 2001). Furthermore, in normal mice, a prolonged fasting period decreases serum RSTN levels, while re-feeding reverses these levels. Interestingly, RSTN mRNA and protein have also been found in the mouse hypothalamus and, *in vitro*, RSTN activates neurones of this region (Morash *et al.* 2002), a very important area for food intake regulation.

The first report of RSTN involvement in feeding behaviour was from our group at the annual meeting of the Society for the Study of Ingestive Behavior in Cincinnati, USA (Brugnoli *et al.* 2004); we showed that recombinant rat RSTN produced by our group inhibited food intake in food-deprived rats. This effect was confirmed and extended, in a full publication, by another group using mouse-derived RSTN (Tovar *et al.* 2005). Interestingly, when ICV is injected, it produces marked changes at the level of neuropeptide gene expression such as agouti-related protein, neuropeptide Y (NPY), and cocaine and amphetamine-regulated transcript, all peptides involved in the regulation of food intake (Vazquez *et al.* 2008).

It is well known that INS also suppresses food intake when injected into the brain (Air *et al.* 2002), while it reduces production of peripheral glucose (Obici *et al.* 2002). On the other hand, hypothalamic injection of RSTN stimulates glucose production (GP; Muse *et al.* 2007).

Studies on feeding behaviour have shown so far that these two peptides given alone at the central level inhibit feeding behaviour (Brugnoli *et al.* 2004, Tovar *et al.* 2005), while studies of central control of GP have demonstrated that they have the opposite effect on GP at the hypothalamic level (Obici *et al.* 2002, Muse *et al.* 2007).

Of particular note for the present study are the reports that both INS (Schwartz *et al.* 1991, 1992) and RSTN (Vazquez *et al.* 2008) have been shown to down-regulate mRNA expression of NPY, a brain neuropeptide implicated in the metabolic regulation of food intake (Kalra *et al.* 1988).

Based on these observations, the present work hypothesized that both INS and RSTN might inhibit food intake through the same mechanism, and a simple addition of their effect on the inhibition should be seen when injected together at the same time into the brain.

The importance of studying the mechanism involved in RSTN food intake control is seen in the work of several authors, who have shown modulation of plasma RSTN levels in rodents (Steppan *et al.* 2001) and humans (Norata *et al.* 2007), respectively, in conditions of diet-induced obesity and in the metabolic syndrome.

Materials and methods

Sixty-one male Wistar rats, weighing 250–300 g (Charles River, Calco, Italy), were individually housed in hanging stainless steel cages with grid floors at constant room temperature (25 ± 1 °C) and humidity (60 ± 5%) with an artificial 12 : 12 h light/dark cycle (dark onset at 08:00 hours). They were offered free access to chow pellets and tap water.

Rats were food deprived overnight and anaesthetized by intramuscular injection of tiletamine hydrochloride (200 mg kg^{-1}) and zolazepam hydrochloride (200 mg kg^{-1}) (Zoletil; Laboratoires Virbac, Carros, France). A prophylactic dose of 25 000 IU benzyl penicillin and 10 mg dihydrostreptomycin (Rubrocillin; Farmaceutici Gellini Spa, Aprilia, Italy) was injected intramuscularly. A 22-gauge guide cannula (12.5 mm long) for intracerebroventricular (ICV) injections was then stereotaxically implanted into the third brain ventricle, 1 mm posterior to the bregma, 1 mm lateral to the sagittal suture and 7.5 mm ventral from the surface of the skull with an angle of 10° (Paxinos & Watson 1986). A stainless steel obturator of the same length was placed into the guide cannula at the end of surgery. A 30-gauge injector 2.5 mm longer than the guide cannula was used for ICV injections. Experiments were carried out at least 1 week after surgery. At the end of each experiment, 1 μL of Indian ink was injected into the third ventricle to verify the position of the cannula tip. In the central injection studies three different groups of animals were used.

Expression vector for recombinant rat RSTN production was prepared by RT-PCR amplification of RSTN mRNA from rat adipose tissue using the following primers: forward: 5′-CGCGGATCCCACGAGG GAGTTGTGCCCTGCTGAG-3′ and reverse: 5′-CGCG GATCCGAGGAACCAACCCGCAGGGTACAGCAG-3′ and cloned in the *Bam*H1 site of the plasmid pTTH8Q1 expression vector (Durocher *et al.* 2002). Plasmid construction and rat RSTN-amplified cDNA were verified by DNA sequencing. Transient transfection of pTTH8Q1-RSTN expression vector in HEK293 SFE cells was performed as described previously (Pham *et al.* 2003). Purification of RSTN was achieved following immobilized-affinity chromatography on TALON beads as indicated by the manufacturer (Clontech: Takara-Bio Europe, Saint-Germain-en-Laye, France). After native elution from the purification column, RSTN purification was controlled on a 4–12% gradient NuPAGE Bis-Tris gel (Invitrogen) using MES buffer (Invitrogen). Before injection, RSTN was dialysed overnight against an 8% NaCl apyrogenic solution at 4 °C. INS 100 UI mL^{-1} HUMULIN was purchased from Lilly (Settimo, Milanese, Italy). Both peptides were

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dissolved in saline solution prior to administration and given in a volume of 2 μL per rat.

Glucose tolerance test

To test the biological activity of our RSTN we replicated the Steppan experiment on glucose tolerance (Steppan *et al.* 2001). Rats were treated twice intraperitoneally (i.p.) with RSTN 0.1 nmol, first, the day before (07:00 hours), and second, 2 h before glucose injection (Steppan *et al.* 2001). Rats were also injected i.p. with a glucose load 2 g kg^{-1} 2 mL^{-1} after an overnight fast. Blood samples obtained from the tail vessels were collected before and 15, 30, 60, 90 and 120 min after the glucose injection. Glucose concentration was immediately determined using a glucose meter (Roche Diagnostic, Rotkreuz, Switzerland).

Effect of ICV injection of RSTN on food-deprived rats

Eighteen rats were food deprived for 12 h (from 08:00 to 20:00 hours). They received an ICV injection of saline or RSTN 0.1, 0.2, 0.3, or 0.4 nmol in a Latin square experimental design. RSTN was injected just before the lights were switched on and food re-presented and recorded for the following 24 h.

Effect of ICV injection of INS on food-deprived rats

The dose of INS chosen to produce a significant reduction in food intake in Wistar rats was based on the literature (Air *et al.* 2002). Nine animals of the same group of animals treated with RSTN were food deprived for 12 h during the dark period of the dark/light cycle (from 08:00 to 20:00 hours). They received an ICV injection of saline, or 10 or 20 mUI of INS in a Latin square experimental design. The central administration was performed at 14:00 hours, 6 h before access to food. Their food intake was then recorded for the following 24 h, precisely 30 min, 1, 2 and 24 h after food was re-presented.

Effect of ICV injection of INS plus RSTN on food-deprived rats

Based on previous experiments, the dose of 10 mUI of INS and the dose of 0.4 nmol of RSTN were chosen to test whether RSTN could increase INS-induced hypophagia. Another group of animals (28) was food deprived as described earlier. They were divided into four groups that received the following treatment: (1) saline + saline, (2) INS + saline, (3) RSTN + saline and (4) INS + RSTN. INS was administered 6 h before the ending of the dark period (14:00 hours) whereas RSTN was administered just before the beginning of the light period (20:00 hours). Food intake was recorded 30 min, 1, 2 and 24 h after RSTN injection.

Effect of resistin on NPY-induced food intake

It has been shown that RSTN reduces mRNA of NPY (Vazquez *et al.* 2008). Based on our first data on RSTN (Brugnoli *et al.* 2004) and this later observation, eight rats were injected centrally with NPY (3 μg/rat) during the light period of the dark/light cycle and their food intake was measured after 30 min, 1, 2 and 24 h. The same group was injected with RSTN (0.2 nmol) a minute before NPY in a Latin square experimental design (Brugnoli *et al.* 2004).

Statistical analysis

The results are expressed as mean ± SEM. The statistical analysis for all the experiments was carried out by ANOVA (repeated measures within subject were taken for central injection animals, while repeated measures between subjects were taken for animals that received peripheral injection), followed by the Newman–Keuls test. $P < 0.05$ was taken as significant.

Results

Glucose tolerance test

Intraperitoneal injection of RSTN into hyperglycaemic rat led to significantly higher glucose concentrations at 90 min (6.6 ± 0.3 mmol L^{-1} vs. 6 ± 0.2 mmol L^{-1} in controls, $P = 0.0006$) and at 120 min (5.9 ± 0.2 mmol L^{-1} vs. 5.2 ± 0.1 mmol L^{-1}, $P = 0.0017$) after RSTN injection (Fig. 1).

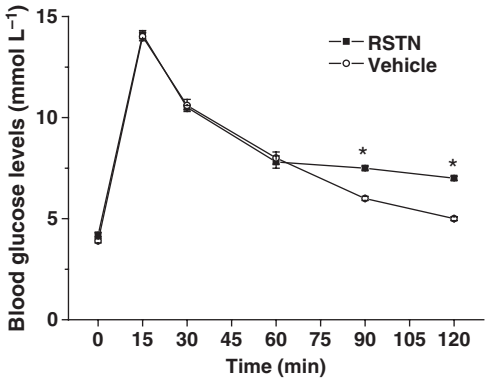

Figure 1 Effect of resistin (RSTN) 0.1 nmol on plasma glucose during glucose tolerance test. Vehicle ($n = 7$, circle) and RSTN-treated rats ($n = 7$, square) were injected i.p. with a glucose load (2 g kg^{-1} 2 mL^{-1}). *$P < 0.05$ for Newman–Keuls test.

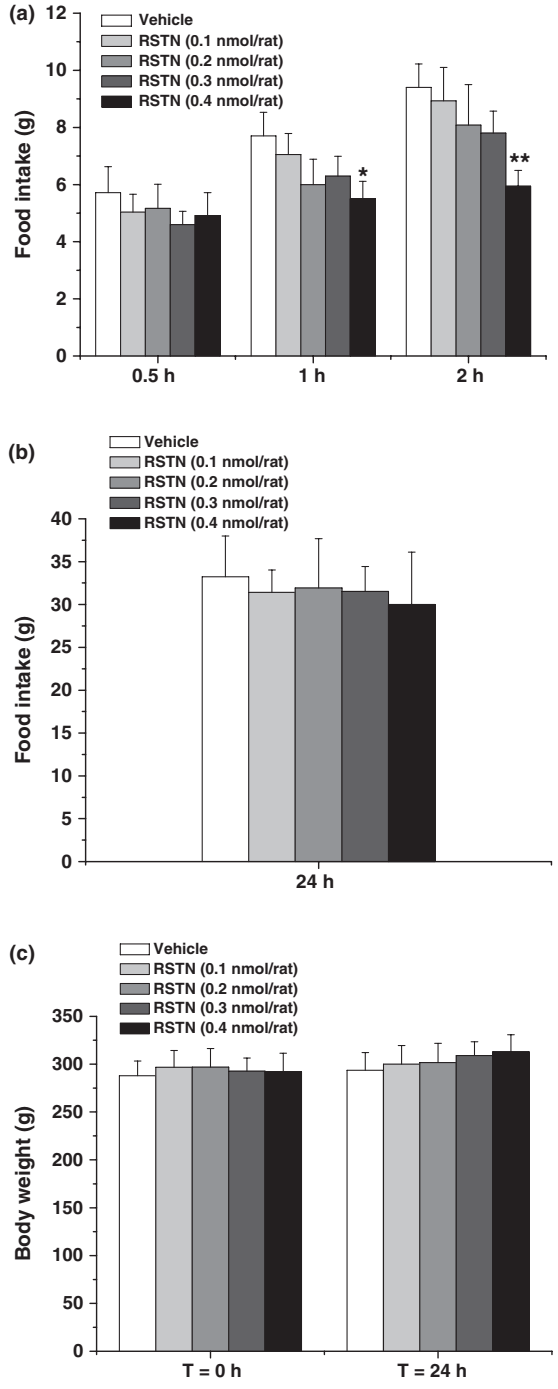

Figure 2 Cumulative 2 h (a) and 24 h (b) food intake (grams) and body weight (c) of 12 h food-deprived rats that received an injection into the third brain ventricle of resistin (RSTN) 0.1, 0.2, 0.3 and 0.4 nmol per rat or its vehicle. Each bar represents the mean ± SEM of nine rats. Statistical difference from controls: $*P < 0.05$; $**P < 0.001$.

Effect of ICV injection of RSTN on food-deprived rats

The ANOVA revealed that central injections of 0.1, 0.2, and 0.3 nmol did not produce any significant reduction in food intake; on the other hand, the dose of 0.4 nmol of RSTN produced a statistically significant reduction in food intake at 1 and 2 h after access to chow pellets [$F(2,16) = 5.791$; $P = 0.012$]. In addition, the ANOVA did not reveal any significant effect after 24 h (Fig. 2).

Effect of ICV injection of INS on food-deprived rats

The ANOVA revealed that central injections of 10 and 20 mUI of INS produced a significant reduction in food intake at 1 and 2 h after the animals were given access to food [$F(2,16) = 10.60$; $P = 0.001$], whereas their intake was not significantly different from controls after 24 h access to food [$F(2,16) = 8.00$; $P = 0.101$] (Fig. 3).

Effect of ICV injection of INS plus RSTN on food-deprived rats

While the combined RSTN and INS injection did suppress food intake in these animals, no further inhibition by the combined compound treatment, compared to either compound alone, was observed. Indeed, the ANOVA confirmed that both treatments produced a significant reduction in 2 h food intake [$F(3,24) = 3.167$; $P = 0.043$] (Fig. 4).

Effect of RSTN on NPY-induced food intake

The ANOVA revealed that central injection of RSTN produced a significant reduction in NPY-induced food intake with a delay of 30 min [$F(3,21) = 91.9$; $P < 0.001$] (Fig. 5).

Discussion

The data of our first experiment show that the rat-derived RSTN produced in our laboratory is biologically active and induces hyperglycaemia in rats, confirming the hyperglycaemic effect of RSTN already observed in the mouse (Steppan *et al.* 2001).

Secondly, both INS and RSTN significantly reduced feeding behaviour in food-deprived rats. It is well known that NPY is involved in the expression of food intake that occurs after a food deprivation period both in normal rats and in genetically obese rodents (Brady *et al.* 1990). The possibility that both peptides might produce their effect on food intake through a common effector such as NPY is supported by the fact that both of them down-regulate mRNA for NPY in the hypothalamus (Schwartz *et al.* 1991, 1992, Vazquez *et al.* 2008). Of interest is the result of our experiment on NPY-induced feeding, in which RSTN produced a significant reduction even for so short a period. Further, the central combination of these two peptides did not show any synergistic effect on feeding behaviour, which

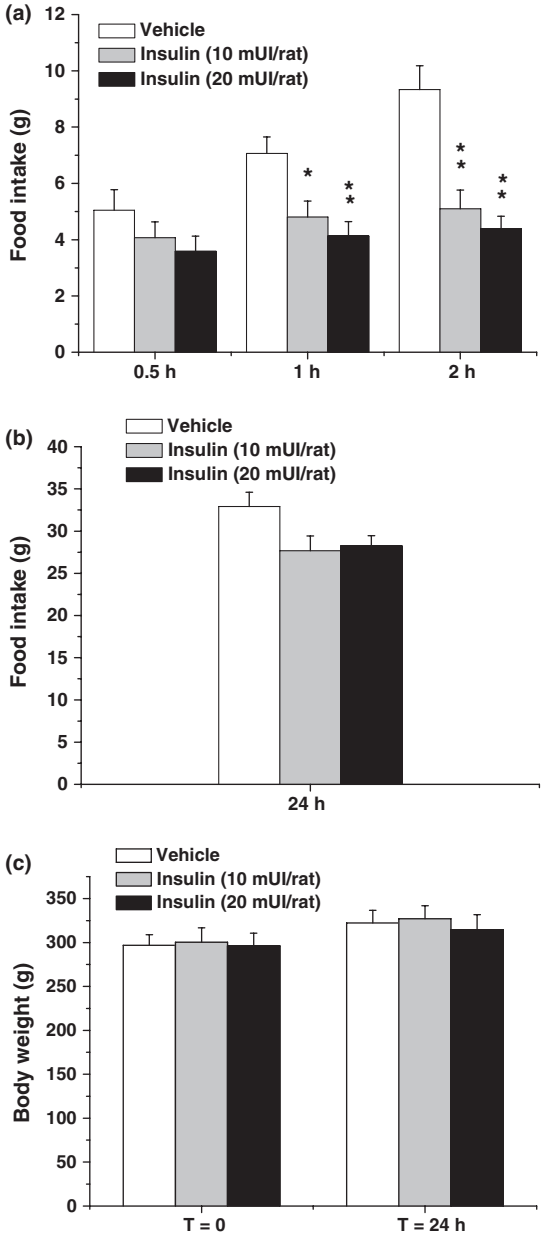

Figure 3 Cumulative 2 and 24 h (a, b) food intake (grams) and body weight (c) of 12 h food-deprived rats that received an injection into the third brain ventricle of either insulin at 10 or 20 mUI per rat, or vehicle. Each bar represents the mean SEM of nine rats. Statistical difference from controls: $^{*}P < 0.05$; $^{**}P < 0.001$.

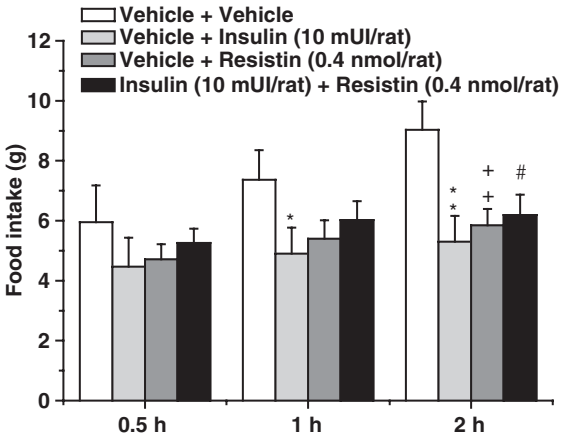

Figure 4 Cumulative 2 h food intake (grams) of 12 h food-deprived rats that received an injection into the third brain ventricle of insulin (INS) 20 mUI, 6 h before the injection of resistin (RSTN) 0.4 nmol or its vehicle (Veh). Each bar represents the mean ± SEM of six Vehicle + Vehicle, six Vehicle + INS, seven Vehicle + RSTN and nine INS + RSTN rats. Statistical difference: Veh + Veh vs. Veh + INS $^{*}P < 0.05$, $^{**}P < 0.01$; Veh + Veh vs. Veh + RSTN $^{+}P < 0.01$; Veh + Veh vs. INS + RSTN $^{\#}P < 0.01$.

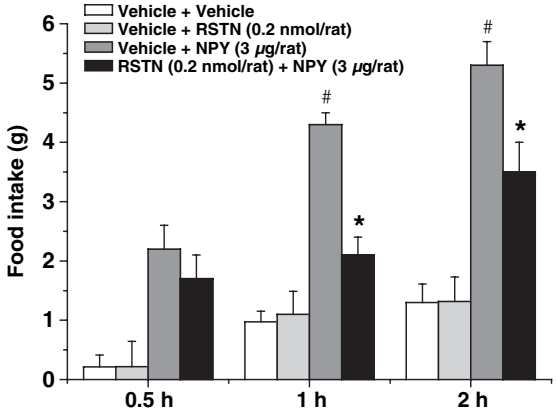

Figure 5 Cumulative food intake (grams) of rats that received an intracerebroventricular injection of either Vehicle (VEH) + Veh, Veh + Resistin (RSTN) 0.2 nmol, Veh + Neuropeptide Y (NPY) 3 μg or RSTN 0.2 nmol + NPY 3 μg. Each bar represents the mean ± SEM of eight rats. Statistical difference: Veh + Veh vs. Veh + NPY $^{\#}P < 0.05$; Veh + NPY vs. RSTN + NPY $^{*}P < 0.05$.

might suggest again the presence of a common effector in the expression of their biological effect.

On the other hand, in our models we did not observe any sum of effects in reducing food intake. A possible explanation why RSTN fails to interfere with INS-induced feeding reduction may be the timing of the RSTN and INS injections. Another explanation why RSTN does not seem to potentiate INS-induced hypophagia could involve the issue of maximum food reduction. INS reduces total food intake by about 30%, whereas RSTN lessens feeding by about 30–40%. Therefore, it is possible that once INS has lowered food intake this much, any further effect of RSTN would be negligible. This further supports the hypothesis that a common effector is involved in the inhibition of food intake.

On the other hand, data on GP after central injection of RSNT and INS alone suggest a competitive action at the hypothalamic level. Indeed, it has recently been demonstrated that central infusion of RSTN alone stimulated GP independently from changes in circulating levels of glucoregulatory hormones (Muse *et al.* 2007), and the infusion of INS alone in the third cerebral ventricle suppressed GP independently from circulating levels of INS and of other glucoregulatory hormones. This suggests an opposite effect of INS, but specific to peripheral GP. Our present results and previous observations on feeding behaviour from other authors have shown that both peptides reduce food intake, suggesting that other hypothalamic structures might be involved in the regulation of GP.

Our data, therefore, strongly suggest that RSTN behaves as a satiety peptide. In this direction, it is interesting to observe the discovery of an RSTN-like peptide expressed in the gut (Rajala *et al.* 2003) and that RSTN levels correlate with energy intake (Steppan *et al.* 2001).

Conflict of interest

The authors declare no conflict of interest.

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