Resistin Regulates Pituitary Somatotrope Cell Function through the Activation of Multiple Signaling Pathways

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The adipokine resistin is an insulin-antagonizing factor that also plays a regulatory role in inflammation, immunity, food intake, and gonadal function. Although adipose tissue is the primary source of resistin, it is also expressed in other tissues and organs, including the pituitary. However, there is no information on whether resistin, as described previously for other adipokines such as leptin and adiponectin, could regulate this gland. Likewise, the molecular basis of resistin actions remains largely unexplored. Here we show that administration of resistin to dispersed rat anterior pituitary cells increased GH release in both the short (4 h) and long (24 h) term, decreased mRNA levels of the receptor of the somatotrope regulator ghrelin, and increased free cytosolic Ca^{2+} concentration in single somatotropes. By means of a pharmacological approach, we found that the stimulatory action of resistin occurs through a Gs protein-dependent mechanism and that the adenylate cyclase/cAMP/protein kinase A pathway, the phosphatidylinositol 3-kinase/Akt pathway, protein kinase C, and extracellular Ca^{2+} entry through L-type voltage-sensitive Ca^{2+} channels are essential players in mediating the effects of resistin on somatotropes. Taken together, our results demonstrate for the first time a regulatory role for resistin on somatotrope function and provide novel insights on the intracellular mechanisms activated by this protein. **(*Endocrinology* 150: 4643–4652, 2009)**

Resistin is an adipose-secreted 12.5-kDa protein that was originally named for its ability to induce insulin resistance (1). Accordingly, systemic administration or transgenic overexpression of resistin in rodents impairs glucose tolerance and hepatic insulin sensitivity, whereas mice lacking the protein show improved insulin sensitivity (reviewed in Ref. 2). Moreover, both genetically and diet-induced obese animals, which frequently display insulin resistance or type 2 diabetes, exhibit increased circulating resistin levels (1). Thus, resistin has been proposed to link obesity with insulin resistance and diabetes in rodents, whereas a similar role for this protein in human remains controversial.

Besides its effects on glucose metabolism and insulin sensitivity, resistin, which is expressed in a variety of tissues and cell types other than adipose (3), regulates a plethora of distinct functions through its action on multiple cell targets, in both rodents and human. Thus, resistin has been

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Abbreviations: AC, Adenylate cyclase; AdipoR, adiponectin receptor; FBS, fetal bovine serum; GHRH-R, GHRH receptor; GHS-R, GH secretagogue receptor; GTPγS, guanosine γ thiophosphate; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; VSCC, voltage-sensitive Ca^{2+} channel.

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shown to exert proinflammatory changes in vascular endothelium (4), promote vascular smooth muscle cell proliferation, stimulate *in vitro* angiogenesis (5), induce proinflammatory cytokine release by macrophages (6) and adipocytes (7), increase testicular testosterone secretion (8), inhibit feeding, and reduce body weight through its action on the hypothalamus (9, 10). Despite all this research on resistin actions, the receptor(s) mediating its biological effects has not yet been identified, and little is known on the intracellular signaling pathways activated by this protein. However, several recent reports indicated that the phosphatidylinositol 3-kinase (PI3K)/Akt (11–14) and MAPK/ERK pathways (11, 15) may be involved in mediating the effects of resistin in certain cell types.

Similar to that reported for resistin, other adipose-derived hormones (or adipokines) such as leptin and adiponectin regulate metabolism and energy homeostasis and also exert a variety of other functions (16, 17). In fact, data have accumulated supporting that resistin, leptin, and adiponectin act as integrators of metabolism with other key homeostatic functions. In this regard, the pituitary gland is fundamental for the integration of several regulatory systems (18). Accordingly, leptin and adiponectin, which are expressed in the human and rodent gland (19, 20), regulate both GH and LH release by the anterior pituitary as well as the expression of several pituitary receptors (20–22). Interestingly, resistin mRNA and protein have been detected in the mouse hypothalamus and pituitary gland (23). However, the potential effects of resistin on the anterior pituitary have never been examined. The aim of the present study therefore was to determine whether, as leptin and adiponectin, resistin might contribute to the regulation of the anterior pituitary and, especially, somatotropes. To this end, the effects of resistin on GH release and the expression of key somatotrope receptors, namely the GHRH receptor (GHRH-R) and the ghrelin/GH secretagogue receptor (GHS-R), were assessed in rat pituitary cell cultures. Furthermore, we explored the contribution of different signaling pathways to the response to resistin by means of a pharmacological approach. Our results indicate that resistin may play an important role in the regulation of somatotrope activity and unveil novel intracellular signals mediating the biological actions of this adipokine.

Materials and Methods

Materials

Tripure reagent was from Invitrogen (Paisley, UK). PowerScript reverse transcriptase was from CLONTECH Laboratories (Palo Alto, CA). iCycler iQ real-time PCR detection system was from Bio-Rad Laboratories (Hercules, CA). Monkey antirat GH, rabbit antirat β-LH, rabbit antirat prolactin, and rabbit antirat ACTH were from the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD), and rabbit monoclonal antiphospho-Akt (Ser473) and rabbit anti-Akt were from Cell Signaling Technology, Inc. (Danvers, MA). Fetal bovine serum (FBS) was from Sera-Lab Ltd. (Crawley Down, UK). Fura-2AM, Pluronic F127, antimonkey fluorescein isothiocyanate-conjugated antiserum, and antirabbit Alexa594-conjugated antiserum were from Molecular Probes (Eugene, OR). [^{3}H]cAMP assay kit was from Amersham Biosciences (Aylesbury, UK). Mouse resistin was from Phoenix Pharmaceuticals Inc. (Belmont, CA). MDL-12,330A and U-73122 were from Research Biochemicals International (Natick, MA), and H89 and phloretin were from Calbiochem Corp. (San Diego, CA). DMEM, antirabbit horseradish peroxidase-conjugated antiserum, thapsigargin, nifedipine, wortmannin, LY-294002, cholera toxin, pertussis toxin, guanosine γ thiophosphate (GTPγS) and all other reagents were from Sigma Chemical Co. (London, UK), unless otherwise specified.

Animals and cell culture

The animal procedures were approved by the Córdoba University Ethical Committee for animal experimentation. Adult male Sprague Dawley rats (Harlan Ibérica, Barcelona, Spain), in 12-h light, 12-h dark cycle, were fed standard rat chow and water *ad libitum*. Pituitary glands were immediately removed after decapitation, the posterior lobes were discarded, and the anterior lobes were enzymatically dispersed (trypsin/collagenase/EDTA) as described (24).

For secretion experiments, dispersed anterior pituitary cells were plated at a density of 300,000 cells onto 24-well culture plates (GIBCO-BRL, Grand Island, NY). For cAMP and Akt phosphorylation measurements, cells were plated at a density of 2×10^6 cells onto 12-well culture plates (GIBCO-BRL). For intracellular free calcium concentration ($[Ca^{2+}]_i$) measurements, cells were plated onto microgrid coverslips (Bellco Glass Inc., Vineland, NJ) at a density of 50,000 cells/coverslip. In all cases, cells were incubated in DMEM supplemented with 10% FBS and 0.1% gentamicin sulfate at 37 C in a 5% CO_2 atmosphere.

Secretion experiments

On d 3, the medium was removed and cells were preincubated in 1 ml serum-free DMEM for 2 h to stabilize basal hormone secretion. Medium was then replaced with fresh DMEM alone or containing resistin at doses ranging from 10^{-14} to 10^{-6} M, and cells were incubated for either 4 or 24 h at 37 C. In another set of experiments, we explored the contribution of different intracellular signaling routes to resistin response by incubating cells for 4 h with 10^{-6} M resistin in the presence of specific blockers of different enzymes or Ca^{2+} channels. Specifically, MDL-12,330A (10^{-6} M), H89 (10^{-6} M), U-73122 (10^{-5} M), phloretin (10^{-6} M), wortmannin (10^{-6} M), and LY-294002 (10^{-5} M) were used to inactivate adenylate cyclase (AC), protein kinase A (PKA), phospholipase C (PLC), protein kinase C (PKC), and PI3K, respectively. In addition, the effect of the selective blocker of L-type voltage-sensitive Ca^{2+} channel (VSCC) nifedipine (10^{-6} M) as well as that of the endoplasmic reticulum Ca^{2+}-ATPase pump inhibitor thapsigargin (0.5×10^{-7} M) was also tested. Inhibitors were added to the incubation medium at the concentrations indicated 2 h before resistin challenge.

Medium samples were collected at the end of the experiments and stored at −20 C until assayed for GH by RIA as described

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(20). Cells in the culture plates were processed for RNA extraction as indicated below.

Real-time quantitative RT-PCR

Total RNA from cells was isolated using Tripure reagent and reversed transcribed using random hexamers and PowerScript reverse transcriptase. Real-time RT-PCR was performed using the iCycler iQ real-time PCR detection system and iQ SYBR green Supermix (Bio-Rad). Reactions were performed using specific primers for rat ghrelin/GHS-R, GHRH-R, and adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2) as described previously (20). Rat hypoxanthine guanine phosphoribosyl transferase (HPRT) was used for internal control.

cAMP measurements

Intracellular cAMP accumulations were measured from anterior pituitary cell cultures treated or not with 10^{-6} M resistin for 30 min as previously described (25).

Akt phosphorylation measurements

After a 30-min incubation period in the absence or presence of 10^{-6} M resistin, cultured cells were lysed in sodium dodecyl sulfate-dithiothreitol sample buffer (62.5 mM Tris-HCl, 2% sodium dodecyl sulfate, 20% glycerol, 100 mM dithiothreitol, and 0.005% bromophenol blue). Whole-cell lysate proteins were separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose membranes. Membranes were stained with Ponceau S for visualization of protein bands and confirmation of equal protein loading for further comparative analysis. Then, membranes were sequentially incubated with rabbit antiphospho-Akt (Ser473) or total Akt (1:1000) and peroxidase-conjugated antirabbit IgG (1:2000). Proteins were visualized using an enhanced chemiluminescence detection system (GE Healthcare, Chalfont St. Giles, UK). Densitometric analysis of the bands was carried out with ImageJ software (National Institutes of Health, Bethesda, MD). Quantitative data from the immunoreactive bands revealed with the anti-phospho-Akt serum were normalized against the corresponding total Akt values.

$[Ca^{2+}]_i$ measurements

Cells were loaded for 30 min at 37 C with 2.5 μM fura-2AM and Pluronic F127 (0.02%) in phenol red-free DMEM containing 20 mM $NaHCO_3$ (pH 7.4). Coverslips were washed with phenol red-free DMEM and mounted on the stage of an Eclipse TE2000-E microscope (Nikon, Tokyo, Japan) equipped with a back-thinned charge-coupled device cooled digital camera (ORCA-BT-1024G; Hamamatsu Photonics, Hamamatsu, Japan), and cells were examined under a ×40 oil immersion objective. Cells were then sequentially exposed to 340 and 380 nm every 5 sec, and fluorescent emission was captured at 505/510 nm before (basal line) and after addition of 10^{-6} M resistin alone or in combination with the following substances: 10^{-6} M GTPγS, 500 nM cholera toxin, 100 nM pertussis toxin, 10^{-6} M MDL-12,330A, 10^{-6} M H89, 10^{-6} M U-73122, 10^{-6} M phloretin, 0.5×10^{-7} M wortmannin, 10^{-6} M nifedipine, 2 mM $CoCl_2$, or 0.5×10^{-7} M thapsigargin. Cells were incubated in the presence of the blockers 30 min before resistin administration, except for thapsigargin as well as cholera toxin and pertussis toxin, which were added to the incubation medium 45 min and 18 h, respectively, before the administration of the adipokine. To confirm that the compounds tested specifically and effectively blocked the desired component of the corresponding signaling pathway without altering other transduction routes, the effects on Ca^{2+} dynamics of pituitary stimulators acting through well-established signaling pathways [GHRH (10^{-6} M) for the AC/PKA route, and GnRH (10^{-6} M) for the PLC/PKC route] were evaluated in cell cultures in parallel to those challenged with resistin.

Imaging acquisition and fluorescence ratio (F340:F380) calculation were carried out using MetaFluor Software (Imaging Corp., West Chester, PA).

Immunocytochemistry

After $[Ca^{2+}]_i$ measurements, cells were fixed in Bouin's fixative. Cells were exposed overnight at 4 C to 1:1000 antirat GH, antirat β-LH, antirat prolactin, or antirat ACTH to identify somatotropes, gonadotropes, lactotropes, and corticotropes, respectively. Immunofluorescence staining with fluorescein isothiocyanate- and Alexa594-conjugated secondary antibodies was accomplished in successive incubations. Coverslips were examined under a Nikon Eclipse TE2000-E microscope, and recorded cells were localized on the alphanumeric grid of coverslips. As negative controls, primary antibodies were omitted.

Statistical analysis

For hormone quantification, mRNA measurements, cAMP determinations, and Akt phosphorylation measurements, a minimum of three replicate wells per treatment were tested in each experiment. Samples from all groups within an experiment were processed at the same time. Each treatment was repeated at least three times on different pituitary cell preparations. A one-way ANOVA followed by a statistical test for multiple comparisons (Duncan's multiple range test and critical ranges) were applied to compare experimental treatments. Data are expressed as the mean ± SEM of the number of experiments indicated in each figure. For Ca^{2+} experiments, a paired Student's t test was used. Results are expressed as mean ± SEM of the number of cells measured in at least three separate experiments. Statistical analysis was assessed by the program Statistica for Windows (Statsoft Inc., Tulsa, OK). Differences were considered significant at $P < 0.05$.

Results

Effect of resistin on hormone release in rat pituitary cell cultures

We first examined the effect of increasing doses of resistin (10^{-14} to 10^{-6} M) on GH release by rat anterior pituitary cell cultures exposed to the adipokine for 4 or 24 h. As shown in Fig. 1A, resistin stimulated basal GH release in 4 h-treated cultures at concentrations of 10^{-8} M or greater. To be more specific, the two highest doses of resistin tested (10^{-8} and 10^{-6} M) evoked significant, maximal increases of GH secretion ($P < 0.05$ *vs.* untreated cultures). In cultures exposed to the adipokine for 24 h, all resistin doses tested caused a similar, significant stimulation of GH release ($P < 0.05$ *vs.* untreated cultures) (Fig. 1B). The EC_{50} values obtained were 3.5×10^{-9} M and 2.6×10^{-12} M for 4 h- and 24 h-treated cultures, respectively.

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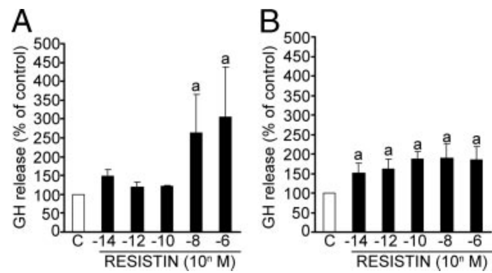

FIG. 1. Effect of resistin on GH release from cultured rat anterior pituitary cells. After 3 d of culture in DMEM-FBS, cells were equilibrated for 3 h in serum-free DMEM and then incubated in the absence [control (C)] or presence of resistin (10^{-14} to 10^{-6} M) for 4 h (A) or 24 h (B). At the end of the incubations, culture media were recovered and GH release was evaluated by RIA. Data are expressed as a percentage of basal values in control cultures (100%, 20.41 ± 4.4 ng GH/ml, 2471 ± 118.9 ng GH/ml, A and B, respectively), and they are the mean (± SEM) from three separate experiments. At least three replicate wells were evaluated per treatment in each experiment. a, $P < 0,05$ *vs.* corresponding control.

In contrast to that found for GH release, treatment of cell cultures with resistin did not modify basal LH release at any of the doses or time of exposure tested (data not shown).

Effect of resistin on the expression of pituitary receptors

We also evaluated the effects of resistin on pituitary mRNA levels of the receptors for the two main stimulators of somatotropes, ghrelin/GHS-R and GHRH-R, by real-time PCR. Thus, when administered for 4 h, resistin significantly decreased ghrelin/GHS-R mRNA levels over a range of 10^{-12} to 10^{-6} M ($P < 0.05$ *vs.* untreated cultures) (Fig. 2A), whereas the adipokine did not modify GHRH-R mRNA levels at any of the doses tested (Fig. 2B). In long-term treated cultures, no significant changes in ghrelin/GHS-R transcript content were observed, and only a single dose of the adipokine (10^{-8} M) modified GHRH-R mRNA levels (see supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Resistin also regulated the expression of the two adiponectin receptors, AdipoR1 and AdipoR2, in rat pituitary cell cultures. Nevertheless, whereas AdipoR1 mRNA expression was down-regulated in response to a wide range of resistin concentrations in both short- and long-term treated cultures, AdipoR2 transcript content was diminished only after short-term exposure to 10^{-8} and 10^{-10} M resistin ($P < 0.05$ *vs.* untreated cultures) (Fig. 2, C and D, and supplemental Fig. S1).

Involvement of the AC/cAMP/PKA pathway in the secretory response of somatotropes to resistin

Once we found that resistin increased GH release, we next determined whether the main signaling pathway

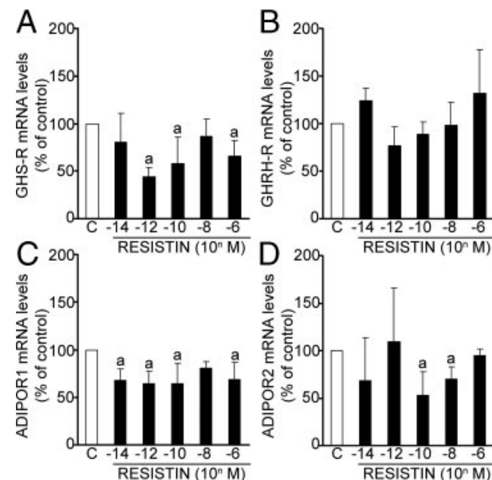

FIG. 2. Effect of resistin on mRNA levels of ghrelin/GHS-R (A), GHRH-R (B), AdipoR1 (C), and AdipoR2 (D) in rat pituitary *in vitro*. After 3 d of culture, dispersed rat pituitary cells were incubated in medium alone [control (C)] or in the presence of resistin (10^{-14} to 10^{-6} M) for 4 h. After culture, cells were harvested and receptor mRNA levels were determined by real-time RT-PCR. Receptor-specific band intensities were determined and adjusted by the signal intensity for hypoxanthine guanine phosphoribosyl transferase. The averaged results were then calculated and expressed as a percentage of vehicle-treated control levels (100%). Data are the mean (± SEM) of three separate experiments. At least three replicate wells were evaluated per treatment in each experiment. a, $P < 0,05$ *vs.* corresponding control.

activated by the hypothalamic somatotrope regulator GHRH, the AC/cAMP/PKA system, could be also involved in the response of somatotropes to the adipokine. This showed that either the specific AC inhibitor MDL-12,330A (10^{-6} M) or the PKA blocker H89 (10^{-6} M), which by themselves did not modify basal hormone secretion, suppressed the stimulation caused by 10^{-6} M resistin on GH release (Fig. 3, A and B). In line with these results, administration of 10^{-6} M resistin to pituitary cell cultures increased cAMP content by 3-fold with respect to that observed in controls (Fig. 3C).

Involvement of the PLC/PKC pathway in the secretory response of somatotropes to resistin

Based on previous reports demonstrating the participation of the PLC/PKC pathway in the response of somatotropes to ghrelin (25), we quantified GH release in response to 10^{-6} M resistin after blockade of these enzymes. As shown in Fig. 3D, the PLC inhibitor U-73122 (10^{-5} M) had no effect on resistin-stimulated GH release. In contrast, the PKC inhibitor phloretin (10^{-6} M) abolished the secretory response of somatotropes to resistin (Fig. 3E). Neither U-73122 nor phloretin affected basal GH release from rat pituitary cell cultures.

Involvement of PI3K in the secretory response of somatotropes to resistin

The possible involvement of PI3K in resistin-induced GH release was assessed by using wortmannin (10^{-6} M) or

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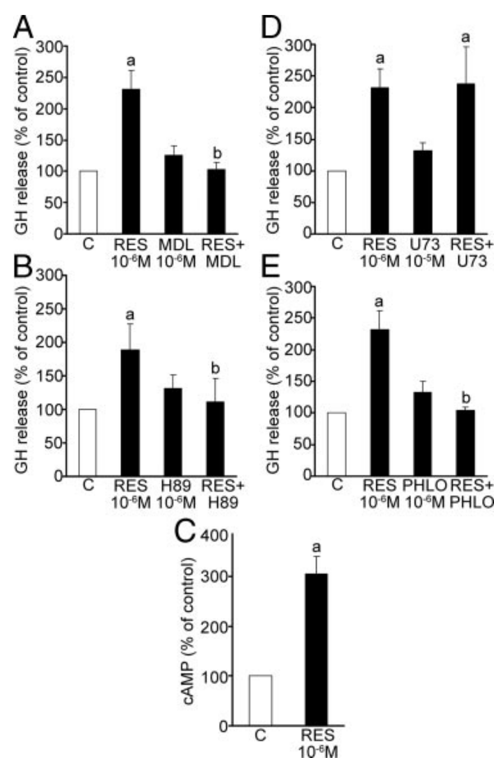

FIG. 3. Effects of inhibition of AC, PKA, PLC, or PKC on resistin-induced GH release and effect of the adipokine on cAMP production. Cultures were treated for 4 h with resistin (RES, 10^{-6} M) alone (C) or in the presence of the AC inhibitor MDL 12,330 A (MDL; 10^{-6} M) (A), the PKA blocker H89 (10^{-6} M) (B), the PLC inhibitor U-73122 (U73; 10^{-5} M) (D), or the PKC blocker phloretin (PHLO; 10^{-6} M) (E), and GH released into the culture medium was evaluated. Inhibitors were added to the incubation medium 2 h before resistin treatment. Data are the mean (± SEM) from at least three separate experiments, each performed in triplicate. a, $P < 0.05$ *vs.* corresponding control (100%; 21.68 ± 10 and 14.3 ± 3.4 ng GH/ml for A and B, respectively; 17.43 ± 1.4 and 21.68 ± 2.5 ng GH/ml for D and E, respectively); b, $P < 0.05$ *vs.* resistin alone. C, For cAMP measurements, cells were equilibrated for 2 h in serum-free medium and then incubated with 1 mM 3-isobutyl-*1*-methylxanthine (IBMX) during 30 min. Then cells were challenged with resistin (10^{-6} M) in the presence of IBMX and incubated for 30 min. Thereafter cAMP production was measured. *Each bar* represents the mean (± SEM) from four independent experiments, each performed in triplicate. Data are expressed as a percentage of the control value (C, 100%; 25.4 ± 3.1 pmol/mg protein). a, $P < 0.05$ *vs.* control.

LY-294002 (10^{-5} M). Results showed that neither wortmannin nor LY-294002 altered basal GH release, whereas they both reduced resistin-induced GH secretion (Fig. 4, A and B). Furthermore, Western blot analysis showed that a 30-min treatment with 10^{-6} M resistin increased Akt phosphorylation by 58% ($P < 0.05$ *vs.* untreated cultures; Fig. 4C).

Contribution of extra- and intracellular Ca^{2+} to the secretory response of somatotropes to resistin

GH secretion was quantified after blockade of extracellular Ca^{2+} entry by use of the L-type VSCC inhibitor nifedipine (10^{-6} M) as well as after depletion of endoplasmic reticulum-associated Ca^{2+} stores by thapsigargin (0.5×10^{-7} M). Neither nifedipine nor thapsigargin modified basal GH release, but whereas nifedipine blocked resistin-induced GH release, thapsigargin did not modify the stimulatory action exerted by the adipokine (Fig. 5, A and B).

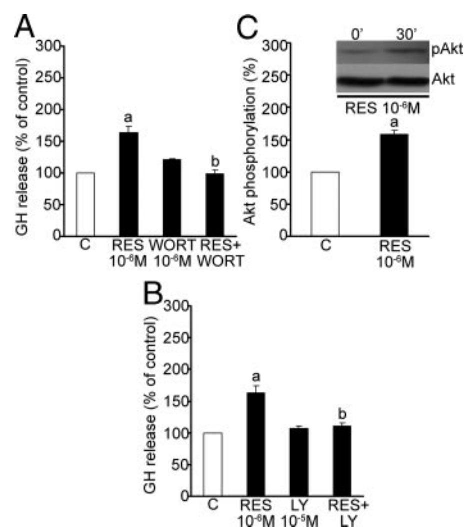

FIG. 4. Secretory response of rat pituitary cells to resistin after blockade of PI3K and effect of the adipokine on Akt phosphorylation. Rat anterior pituitary cells were incubated for 4 h in the absence or presence of wortmannin (WORT; 10^{-6} M) (A) or LY-294,002 (LY; 10^{-5} M) (B), and then GH released was evaluated. Inhibitors were added to the incubation medium 2 h before resistin (RES) treatment. Data are means (± SEM) from three independent experiments, each performed in triplicate. a, $P < 0.05$ *vs.* corresponding control (100%; 27.59 ± 0.96 ng GH per milliliter); b, $P < 0.05$ *vs.* resistin alone. C, Resistin-induced phosphorylation of Akt in rat pituitary cell cultures. Lysates from control and 30 min resistin-treated rat pituitary cells were subjected to Western blotting with Akt and antiphospho-Akt (p-Akt) antibodies. Densitometric analysis of the bands was carried out using ImageJ software. Quantitative data from the immunoreactive bands were normalized against the corresponding control values and represented as ratio of pAkt *vs.* Akt. The data represent the means (± SEM) of three independent experiments. a, $P < 0.05$ *vs.* corresponding control.

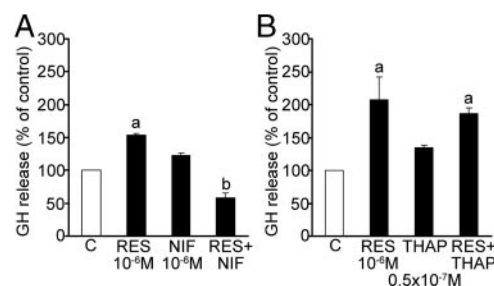

FIG. 5. Contribution of extra- and intracellular Ca^{2+} to the stimulatory effect of resistin on GH release from rat somatotropes. The role of extracellular Ca^{2+} was investigated by incubating rat pituitary cells in the presence of nifedipine (NIF; 10^{-6} M) alone or in combination with 10^{-6} M resistin (RES; A), whereas the participation of Ca^{2+} stores from the endoplasmic reticulum was assessed in cultures of cells treated with thapsigargin (THAP; 0.5×10^{-7} M) alone or together with 10^{-6} M resistin (B). Cells were treated with the corresponding substances for 4 h, and GH release was evaluated thereafter. Data are means (± SEM) from four (for nifedipine) or three (for thapsigargin) experiments, each performed in triplicate (100%; 38.5 ± 1.6 and 27.59 ± 0.96 ng GH per milliliter for A and B, respectively). a, $P < 0.05$ *vs.* control; b, $P < 0.05$ *vs.* resistin alone.

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Effect of resistin on $[Ca^{2+}]_i$ in rat pituitary cells

Administration of resistin (10^{-6} M) induced a substantial rise in $[Ca^{2+}]_i$ in 19.3% of pituitary cells recorded (n = 185 of 959 cells). Specifically, 29.2% of somatotropes (n = 35 of 120 somatotropes) responded to the adipokine. On average, resistin increased by 45.65 ± 4.2% basal $[Ca^{2+}]_i$ in these cells ($P < 0.05$). Detailed analysis of the response evoked by resistin revealed the occurrence of two types of $[Ca^{2+}]_i$ profiles in somatotropes: a peak-type response (42.9% of somatotropes) or a plateau-type response (57.1%) (supplemental Fig. 2). Maximal $[Ca^{2+}]_i$ levels evoked by resistin were of similar magnitude in somatotropes displaying either peak- or plateau-type responses (52.0 ± 14.0 and 45.7 ± 4.0%, respectively, with respect to basal values; $P < 0.05$).

We also investigated the possible effects of resistin on $[Ca^{2+}]_i$ on other pituitary cell types. Resistin increased by 21.41 ± 5% $[Ca^{2+}]_i$ ($P < 0.05$ *vs.* basal values) in 14.3% of lactotropes (n = 6 of 42 cells). A low percentage of corticotropes (16%; n = 4 of 25 cells) showed a 36% decrease in $[Ca^{2+}]_i$ after resistin challenge, although this reduction was not statistically significant ($P = 0.1$). Likewise, only four of the 62 cells identified as LH-containing cells (6.4%) displayed a modest, not significant, decrease in $[Ca^{2+}]_i$ (−8%) in response to resistin. In all the experiments, cells exhibiting Ca^{2+} responses could be immunoidentified *post facto* as belonging to one of the four cell types analyzed herein, thus precluding the analysis of the response of the fifth cell type of the anterior pituitary, thyrotropes, to resistin.

Intracellular signaling mechanisms mediating the stimulatory effect of resistin on $[Ca^{2+}]_i$ in pituitary cells

We first examined the participation of G proteins in resistin-induced effects on $[Ca^{2+}]_i$. As shown in Table 1, pretreatment of cells with the nonhydrolyzable analog GTPγ-S (10^{-6} M) completely blocked the response to 10^{-6} M resistin in the vast majority of cells tested. To identify the nature of the G proteins involved in the effect of resistin, cells were treated with cholera toxin (500 nM) for inactivating G_s or pertussis toxin (100 nM) for inhibiting $G_{i/o}$ and then stimulated with 10^{-6} M resistin. This showed that resistin evoked a low, nonsignificant increase in only 2% of cholera toxin-treated cells, whereas pertussis toxin did not impair the effects of resistin either in terms of percentage of responsive cells (20%) or the amplitude of the $[Ca^{2+}]_i$ increase (57.9 ± 9.4%) (Table 1).

To ascertain the possible contribution of the AC/PKA pathway to resistin-induced $[Ca^{2+}]_i$ increase, cultures were treated with the AC inhibitor MDL-12,330A (10^{-6} M) or the PKA blocker H89 (10^{-6} M). As shown in Table 1, blockade of either enzyme completely blunted the $[Ca^{2+}]_i$ response of pituitary cells to resistin.

TABLE 1. Percentages of responsive rat anterior pituitary cells and $[Ca^{2+}]_i$ increases induced by resistin (10^{-6} M) alone or in the presence (+) of various test substances

Test substance	Responsive cells, %	$[Ca^{2+}]_i$ increase, %
Resistin (10^{-6} M)	19.3 (n = 959)	43.0 ± 6.0[a]
+ GTPγS (10^{-6} M)	1.2 (n = 154)	18.9 ± 1.5[a]
+ Cholera toxin (500 nM)	2 (n = 143)	7.1 ± 1.8
+ Pertussis toxin (100 nM)	20 (n = 189)	57.9 ± 9.4[a]
+ MDL-12,330 A (10^{-6} M)	0 (n = 179)	0
+ H89 (10^{-6} M)	2 (n = 163)	9.2 ± 2.8
+ U-73,122 (10^{-6} M)	6.1 (n = 81)	11.88 ± 6.3
+ Phloretin (10^{-6} M)	12.9 (n = 339)	25.58 ± 2.37[a]
+ Cl_2Co (2 mM)	2.9 (n = 170)	30.6 ± 9.4
+ Nifedipine (10^{-6} M)	3.5 (n = 314)	57.38 ± 12.6[a]
+ Thapsigargin (0.5×10^{-7} M)	21 (n = 317)	30.5 ± 2.2[a]
+ Wortmannin (10^{-6} M)	11.2 (n = 348)	18.38 ± 1.6

Concentrations of resistin and the different inhibitors used and total numbers of cells measured in each case are shown in parentheses. Significant differences ($P < 0.05$) were assessed by one-way ANOVA followed by a *post hoc* Duncan's test.

[a] Compare with basal values.

We also investigated whether the effect of resistin was dependent on the activity of PLC and PKC. In the presence of the PLC inhibitor U-73122 (10^{-6} M), only 6.1% of pituitary cells exhibited a moderate increase in $[Ca^{2+}]_i$ after exposure to resistin. Inactivation of PKC by phloretin (10^{-6} M) caused a partial reduction in the response of cells to resistin. Specifically, 12.9% of cells exhibited significant $[Ca^{2+}]_i$ increases after resistin challenge in the presence of phloretin (25.5 ± 2.3% *vs.* basal values; $P < 0.05$).

With respect to the contribution of extracellular Ca^{2+} to the effect of resistin, treatment of the cultures with 2 mM Cl_2Co caused a significant reduction in the proportion of cells responding to the adipokine (2.9%) (Table 1). Moreover, blockade of L-type VSCC by nifedipine (10^{-6} M) reduced to 3.5% the percentage of responsive cells. In contrast, depletion of intracellular Ca^{2+} pools by thapsigargin (0.5×10^{-7} M) did not modify the percentage of responsive cells (Table 1), which displayed significant $[Ca^{2+}]_i$ increases in response to resistin (30.5 ± 2.2% *vs.* basal values; $P < 0.05$). Finally, the presence of wortmannin (10^{-6} M) in the culture medium induced a modest increase in $[Ca^{2+}]_i$ in 11.2% of pituitary cells.

Discussion

Previous studies demonstrating the expression of resistin in the rodent pituitary and hypothalamus suggested a possible neuroendocrine role for this adipokine (23). In sup-

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port of this notion, here we show that administration of resistin to pituitary cell cultures evoked a three-pronged effect on somatotropes. First, resistin increased GH release in both the short (4 h) and the long (24 h) term. Second, it regulated mRNA expression levels of the receptor mediating the effects of one major stimulator of somatotropes, ghrelin. Finally, the adipokine increased $[Ca^{2+}]_i$ in single somatotropes. When viewed together, these results provide evidence that pituitary somatotropes are a direct target of resistin action.

Interestingly, the EC_{50} values for resistin, determined from the GH release dose-response curve in 4 h-treated cultures, was 3.5×10^{-9} M, which is within the concentration range of circulating resistin in rat (26). Noteworthy, the effect of resistin did not follow a typical dose-response pattern in terms of GH secretion as well as several other parameters evaluated (*i.e.* receptor gene expression), an observation that has been reported for this and other signaling molecules (7, 20, 27, 28). Although the reasons are unclear at present, several possibilities can be put forward. One plausible hypothesis is that, depending on its concentration, resistin might induce different structural conformations of its receptor(s) and/or the selective interaction of resistin receptor(s) with other receptors (*i.e.* homo- or heterodimerization), which in turn might modify resistin activity because it has been reported to occur for other receptors in response to their corresponding ligands (29). Alternatively, it is yet unknown whether the effects of resistin are mediated by one or several receptors because none has been identified. If there is more than one receptor for resistin, it could be possible that they may have different affinities for resistin with different biological effects that could explain the atypical dose-response curves observed herein. Nevertheless, until further data are obtained, these explanations are at present speculative.

We also found that exposure of somatotropes to resistin induced a significant rise in $[Ca^{2+}]_i$ in this cell type. Inasmuch as Ca^{2+} is a key intracellular mediator in GH release by somatotropes (30), these data, together with the results obtained in the secretion experiments, strongly support the view that resistin acts directly on these cells to stimulate GH secretion. Notwithstanding this, the effect of resistin appears to be specific for a somatotrope subpopulation because only 29.2% of somatotropes exhibited significant $[Ca^{2+}]_i$ increases in response to resistin. In this regard, we and other authors have shown that the population of pituitary somatotropes is not homogeneous but that it is in fact composed of two morphologically and functionally distinct somatotrope subtypes. Specifically, we have shown that the two somatotrope subtypes display significant differences in GH-releasing activity both under basal conditions and in response to GHRH and somatostatin (31–33), which, in turn, correlates with their distinct expression patterns of GHRH-R and various somatostatin receptors (1, 2, and 5) (34). Hence, it is conceivable that, as for these receptors, the resistin receptor(s) is selectively (or more abundantly) expressed in a certain subpopulation of somatotropes, thus accounting for the relatively low percentage of somatotropes exhibiting $[Ca^{2+}]_i$ increases on resistin challenge.

Different families of membrane receptors have been reported to signal through the $[Ca^{2+}]_i$ system, including G protein-coupled receptors and tyrosine kinase-linked receptors (35). By using a pharmacological approach, we have shown that resistin-induced $[Ca^{2+}]_i$ increase involves the participation of G proteins. More specifically, Gs proteins, but not pertussis toxin-sensitive Gi/o-proteins, seem to mediate the effect of resistin on somatotropes. Thus, blockade of major downstream effectors of Gs protein-associated signaling pathway, namely AC and PKA, abolished the effect of resistin on both $[Ca^{2+}]_i$ and GH release. These data, together with the observation that resistin increased cAMP levels in the cultures, indicate that the AC/cAMP/PKA transduction pathway is crucial for this protein to exert its stimulatory action on somatotropes and strongly suggest that the interaction of resistin with these cells occurs through a Gs protein-dependent mechanism. To the best of our knowledge, this is the first report demonstrating the involvement of this signaling pathway in mediating the biological action of resistin.

We also observed that blockade of PKC abolished resistin-induced GH release, thus suggesting that this enzyme plays a major role in the secretory response of somatotropes to resistin. However, neither inhibition of PLC nor blockade of Ca^{2+} mobilization from intracellular pools had any effect on the stimulation caused by resistin on GH release. These results suggest that resistin does not primarily act on somatotropes through the canonical Gq protein-associated pathway to enhance hormone secretion. In addition to the AC/cAMP/PKA pathway and PKC, the PI3K/Akt signaling system also seems to mediate resistin-induced effects on both GH release and $[Ca^{2+}]_i$ in somatotropes. Similarly, resistin has been shown to increase PI3K-induced phosphorylation of Akt in other cellular models (11–14), thus pointing out the relevance of this signaling pathway in resistin biological actions. In summary, our results indicate that the overall response of the somatotrope to resistin does not rely on the activation of a single transduction system but encompasses the participation of multiple intracellular signaling events, which might be interconnected through cross talk mechanisms. Interestingly, all the signaling systems involved in resistin-induced effects on GH release by somatotropes have been shown to directly or indirectly act on VSCC channels in

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this or other cell types (36, 37). Inasmuch as blockade of extracellular Ca^{2+} entry through L-type VSCC abolished the effects of resistin on somatotropes and in view of the pivotal role of $[Ca^{2+}]_i$ rise in triggering exocytosis in somatotropes (30), it is reasonable to propose that the multiple signaling pathways activated by resistin in these cells may converge in the activation of these channels to induce GH release.

Resistin also regulated the expression of the receptors for a major stimulator of somatotropes, ghrelin. Specifically, resistin reduced ghrelin/GHS-R expression levels in the cultures. A similar down-regulation of ghrelin/GHS-R mRNA has been described to occur in response to ghrelin as well as GHRH (38, 39). In contrast, resistin only occasionally reduced pituitary GHRH-R mRNA content. A divergent effect on GHRH-R and ghrelin/GHS-R expression has been also reported to occur in response to several stimulators of GH release (38, 40, 41), thus indicating that, although the two receptors converge similar actions on GH release in somatotropes, they are differentially controlled by stimulatory inputs regulating this cell type.

We have recently shown that another adipokine, adiponectin, also acts *in vitro* on the rat pituitary, which indeed expresses the two adiponectin receptors, AdipoR1 and AdipoR2 (20). However, in contrast to what was found herein for resistin, adiponectin inhibited GH release and increased ghrelin/GHS-R and GHRH-R mRNA levels (20). These findings indicate that adiponectin and resistin exert opposite effects on somatotropes. Moreover, we also found that resistin decreased pituitary mRNA content of both AdipoR1 and AdipoR2. Interestingly, these two adipokines also differ in their effects on glucose and lipid metabolism as well as the cardiovascular and immune systems (2, 17, 42). The divergent effects of resistin and adiponectin on GH production, together with the observation that circulating levels of resistin are inversely correlated to those of adiponectin in obese and lean animals (2) or in response to fasting and refeeding (43, 44), support the notion that adipose tissue, through the selective production of adiponectin or resistin, differentially regulates the somatotropic axis according to the metabolic status. On the other hand, similar to resistin, leptin, whose circulating levels parallel those of resistin in response to changes in nutritional status and relation to adiposity (2), stimulates GH release (22), thus suggesting that resistin and leptin may act in a coordinate manner at the pituitary to modulate GH release. In this scenario, the decrease in GH release observed during fasting in rats (45, 46) could be accounted for by, among other factors, the increased adiponectin and decreased resistin and leptin plasma levels associated with this catabolic condition, whereas reversal of the circulating levels of these adipokines after refeeding could contribute to restore GH secretion by the pituitary (45). It should be noted that obese animals, despite their high resistin plasma levels (44), exhibit impaired GH secretion (47). Consistent with this notion, it has been clearly established that GH response to all known stimuli (*i.e.* fasting, GHRH) is severely reduced or blunted in obese animals and humans (48, 49). It is conceivable that the stimulatory effect of resistin on GH release is overridden in the obese state by the action of other somatotrope regulators such as free fatty acids or insulin, whose circulating levels are positively correlated to adiposity and are known to exert a direct inhibitory effect on somatotrope secretion (50, 51).

In summary, we have shown that resistin increases both GH release and $[Ca^{2+}]_i$ in somatotropes and regulates the expression of a key stimulatory receptor of these cells, namely the ghrelin/GHS-R. Moreover, our results indicate that resistin-induced effects on GH release and $[Ca^{2+}]_i$ involve the activation of multiple signaling routes including the AC/cAMP/PKA system, the PI3K/Akt pathway, PKC, and extracellular Ca^{2+} entry through L-type VSCC. Together, these results suggest that circulating resistin may act on somatotropes to increase GH release. Furthermore, the observation that the adipokine is expressed in the pituitary of mouse (23), rat, and human (our unpublished results) supports the view that locally produced resistin may also contribute to regulate somatotropes through a paracrine/autocrine mode of action. In all, these findings suggest a role for resistin, local and/or systemic, in the regulation of GH axis function. Although further experiments are needed to clearly establish the physiological relevance of resistin effect on GH release, it should be noted that it was comparable with that of ghrelin and lower than that of GHRH in the same experimental model (20).

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