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Research Article

Pancreastatin, a chromogranin A-derived peptide, inhibits leptin and enhances UCP-2 expression in isolated rat adipocytes

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Abstract. Leptin, the ob gene product, is an adipocyte-secreted hormone that centrally regulates weight by decreasing caloric intake and increasing energy expenditure. Expression of leptin is regulated by dietary status, insulin, glucocorticoids and catecholamines. Pancreastatin (PST), a chromogranin A-derived peptide, correlates with catecholamine levels, and may play a role in the physiology of stress, modulating endocrine secretion and metabolism. Thus, PST has been found to exert a lipolytic and anti-insulin effect in white adipocytes. The aim of the present work was to investigate a possible role of PST modulating the expression of key genes involved in lipid storage and metabolism: leptin, PPAR-γ2, UCP-1 and UCP-2. We incubated isolated rat epididymal adipocytes with 100 nM PST for 16 and 24 h. Leptin, UCP-2 and UCP-1 mRNA levels were assessed by RT-PCR, followed by Southern blot. Leptin secretion was also measured by ELISA. PST inhibited leptin expression and secretion at 16-h incubation, but this effect was no longer observed after 24 h. On the other hand, PST stimulated the expression of UCP-2 after 16 h. However, the effect was still significant after 24 h. The inhibitory effect of PST on leptin expression and secretion and the stimulation of UCP-2 expression were prevented by blocking PKC. UCP-1 and PPR-γ2 expression did not change after PST stimulation. Leptin differentially regulates the expression of key genes in the rat adipocyte, upregulating the expression of UCP-2 and inhibiting the expression and secretion of leptin by a mechanism that involves PKC activity. These effects may contribute to the metabolic action of catecholamines in physiological and pathophysiological conditions with increased sympathetic activity.

Key words. Chromogranin A; pancreastatin; adipocyte; leptin; UCP; PPAR-γ; adrenergic.

Leptin, the 16-kDa nonglycosylated protein product of the ob gene [1], is a hormone synthesized mainly in adipose cells [2] to regulate weight control in a central manner, via its cognate receptor in the hypothalamus [3], by regulating food intake and energy expenditure [4, 5]. Leptin is released into the circulation, and plasma levels correlate with total body fat mass [6]. Leptin administration to lean or ob/ob (leptin-deficient) mice increases basal metabolic rate and reduces food intake, leading to weight loss [7–9]. This effect of leptin is known to be mediated by modulating sympathetic nervous system activity, which in turn, modulates leptin [10–12]. Thus, adrenergic agonists, in particular those specific for the β_3-adrenergic receptor, have been found to inhibit leptin expression [13–15]. Furthermore, adrenergic nerve activity is well known to regulate adipose tissue energy stores through lipolysis and activation of thermogenesis [16].

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Catecholamines are secreted along with chromogranin A, a glycoprotein very abundant in chromaffin granules, but also present in many neuroendocrine cells [17]. Chromogranin A is the precursor of a variety of peptides with different functions [18]. Pancreastatin (PST) [19] is one of the biologically active peptides derived from chromogranin A [19, 20]. High PST levels have been correlated with catecholamines in hypertension and gestational diabetes, i.e. pathophysiological states with increased adrenergic activity [21, 22]. Thus, PST has been suggested to play a role in the physiology of stress, modulating endocrine secretion and metabolism [23].

The metabolic actions of PST have been thoroughly studied and we already know some of the molecular mechanisms by which PST modulates glucose, lipid and protein metabolism in the adipocyte [22], with a consequence which is generally counterregulatory to that of insulin.

PST has been shown to exert its metabolic effect in rat adipocytes [24] by activating specific receptors [25], which trigger the stimulation of the coupled $G\alpha_{q/11}$-Gi_{1-2} system [25], resulting in activation of the effector system phospholipase C-β-protein kinase C-mitogen-activated protein kinase (PLC-β-PKC-MAPK) [25, 26]. PST has a counterregulatory effect on insulin signaling and action, inhibiting glucose uptake, glycogen and lipid synthesis, as well as promoting lipolysis [24, 26, 27]. Moreover, PST has a negative cross-talk with insulin receptor signaling [26], blocking the phosphatidylinositol 3-kinase (PI3K) signaling cascade at an early stage in the insulin receptor signal transduction mediated by the activation of PKC [26, 28]. Furthermore, PKC activity has been found to directly mediate some metabolic effects of PST, such as the activation of glycogen synthase kinase-3 (GSK-3), providing further mechanisms that explain the inhibition of basal and insulin-mediated glycogen synthesis in rat adipocytes [27].

Mitochondrial uncoupling proteins (UCPs) encode proton carriers that uncouple electron transport from ATP synthesis and are implicated in increases in energy expenditure [29]. UCP-1 is mainly expressed in brown adipose tissue [30], but is also present in white adipocytes [31], whereas UCP-2 is abundant in white adipose tissue [32, 33]. UCPs, as well as leptin expression, have been found to be modulated by β_3 adrenergic activation [16, 31, 34–36] and peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists [37, 38].

Given the functional relationship between catecholamines and PST, we sought to assess the effect of in vitro PST stimulation of isolated rat adipocytes on the expression of key genes involved in lipid metabolism and energy homeostasis: leptin, UCP-1, UCP-2 and PPAR-γ.

Materials and methods

Materials

PST was purchased from Peninsula Laboratories (St. Helens U. K.). The PKC inhibitor (bisindolylmaleimide) and cycloheximide were from Roche Molecular Biochemicals (Barcelona, Spain).

Adipocyte isolation

Adipocytes were prepared from the epididymal fat pads of ad libitum-fed 120- to 180-g male Wistar rats according to the method described by Rodbell [38], with minor modifications [24, 25].

Adipocyte incubation

Adipocytes were incubated in six-well plates in Dulbecco's modified Eagle's medium (DMEM), supplemented with 0.1% bovine serum albumin (BSA), and 2 mM L-glutamine, 100 µU/ml penicillin, and 100 µg/ml streptomycin (complete medium without serum) (all from Gibco, Invitrogen, Barcelona, Spain). Adipocytes were incubated at 37°C in 5% CO_2 atmosphere. When the protein synthesis inhibitor cycloheximide (20 µg/ml), or the PKC inhibitor bisindolylmaleimide (50 nM) were employed, they were added 5 min before the addition of PST, and controls were incubated with vehicle. Samples were taken from the incubation medium at 16 h and 24 h for leptin determination or RNA isolation.

Leptin assay

Leptin levels in cell incubation medium and cell lysates were determined with a commercially available ELISA kit (R&D Systems, Minneapolis, Minn.). Leptin concentrations were within the detection range of the kit (62–4000 pg/ml). All samples were assayed in duplicate. Data are means ± SE from three different experiments. Differences among groups were determined by analysis of variance followed by a post hoc multiple comparison test. A p value <0.05 was considered significant.

mRNA detection by RT-PCR

Primary cultured adipocytes were incubated at 37°C in a 5% CO_2 atmosphere for 16 h and 24 h in the absence or presence of PST (100 nM). Total RNA (1×10^6 cells) was extracted with the QuickPrep Total RNA extraction kit (AmershamPharmacia Biotech, Barcelona, Spain). First-strand cDNA synthesis was performed using oligo-dT primer (kit from Roche Molecular Biochemicals) and used for detection of the leptin, UCP-1, UCP-2 and PPAR-γ messenger RNA (mRNA) by RT-PCR as previously described [35, 39–42]. The sequences of primers and hybridization probes for leptin, UCPs and PPAR-γ are described in table 1. β-Actin mRNA expression was used as an internal control. The samples were amplified for 23–25 cycles using the following parameters: denat-

Table 1. Sequences of PCR primers.

Gene	Sense primer (5′−3′)	Antisense primer (5′−3′)	Internal oligonucleotides (5′−3′)	Size of cDNA (bp)
β-Actin	TTGTAACCAACTGGG-ACGATATGG	GATCTTGATCTTCATGGT-GCTAGG	GGTCAGGATCTTCATGAGGTA-GTCTGTCAG	764
Leptin	GGAGGAATCCCTGCTC-CAGC	CTTCTCCTGAGGATACCTGG-	GGTCTGAGAGGCAGGGAGCA-GCTCTTGGAGAAGGC	640
UCP-1	CAGGCTTCCAGTACTAT-TAGGT	TGCCAGTATGTGGTGGTTC-ACAAG	CGGACTTTGGCGGTGTCCAG-CGGGAAGGTGAT	486
UCP-2	CAGTTCTACACCAAGGG-CTCAGAG	TCTGTCATGAGGTTGGCTT-TCAG	GTCGGAGATACCAGAGCACT-GTCGAAGCCT	323
PPAR-γ	ATAAAGTCCTTCCCGCT-GACCAAAGCC	GCGGTCTCCACTGAGAATA-ATGACAGC	CCACAGGTACTCTAGTAGATGT-GGTACGACCGG	524

uration at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min. The annealing temperature for UCP-2 primers was 65 °C, and 60 °C for β-actin primers. The PCR products were analyzed by 2% agarose gel with ethidium bromide staining and Southern hybridization. The hybridization probe was labeled by tailing the oligonucleotide with digoxigenin-dUTP (kit from Roche Molecular Biochemicals). Specific hybridization was developed with anti-digoxigenin antibodies (Roche Molecular Biochemicals) labeled with peroxidase [40]. The bands were visualized by a high-sensitive chemiluminescence system (SuperSignal; Pierce, Rockfold, Ill.). The bands obtained were scanned and analyzed by the PCBAS2.0 program.

Values are expressed as means ± SE Student's t test was used for comparisons, with differences being considered significant at p < 0.05.

Results

Pancreastatin inhibits the expression and secretion of leptin from isolated rat adipocytes

Adrenergic activation inhibits leptin expression in white adipose tissue [15, 35]. The effect of the chromogranin A-derived peptide PST on the degree of leptin mRNA expression was tested in isolated rat adipocytes by a semiquantitative RT-PCR method. Figure 1 A shows that 16-h exposure to PST significantly decreased leptin expression in cultured rat adipocytes. Thus, 100 nM PST inhibited basal leptin mRNA expression by about 50%. This effect of PST was transient, since no significant differences were found after 24 h incubation.

Since PST reversibly inhibited leptin mRNA expression, we next wanted to assess whether PST would also modify leptin secretion. Thus, we measured leptin production in the incubation medium of adipocytes after 16- and 24-h incubation, by a specific ELISA. Analysis of the PST effect on leptin secretion is shown in figure 1 B. PST
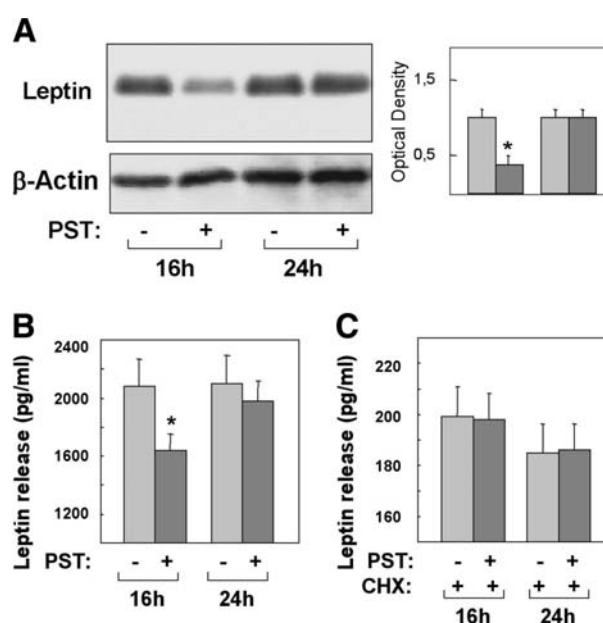

Figure 1. PST inhibits leptin expression and secretion from rat adipocytes. Isolated epididymal rat adipocytes were incubated for 16 and 24 h in the absence or presence of 100 nM PST. (A) Total RNA was obtained and leptin expression was assessed by RT-PCR and Southern blot with an internal oligonucleotide. Leptin bands were quantified and normalized with the β-actin bands. A representative experiment out of four is shown. Densitograms with SE are shown. *p < 0.05. (B) Leptin levels were measured in the incubation medium by ELISA at 16 and 24 h. Data are means ± SE from three independent experiments run in duplicates. * p < 0.05. (C) Leptin levels were measured in the culture medium from adipocytes incubated in the presence of cycloheximide (CHX) (20 μg/ml).

(100 nM) significantly inhibited leptin secretion, decreasing by about 30% the leptin release from control adipocytes. We next employed a protein synthesis inhibitor (cycloheximide, 20 μg/ml) to check a possible effect of PST on the secretory process of synthesized leptin. As shown in figure 1 C, cycloheximide treatment dramatically decreased leptin secretion, as should be expected for the inhibition of protein synthesis. However,

no differences in leptin levels were obtained in medium samples from adipocytes incubated with or without PST, when adipocytes were cultured in the presence of 20 μg/ml cycloheximide. Therefore, this inhibitory effect of PST on leptin secretion seems to be due to the observed decrease in mRNA, rather than to an inhibitory effect on leptin release. Furthermore, we found no accumulation of leptin inside the PST-stimulated adipocytes, as assessed by quantification of leptin levels in cell lysates (data not shown).

PST enhances the expression of UCP-2 in rat adipocytes

Catecholamines, and more specifically β_3-adrenergic agents, increase UCP-2 expression in white adipose tissue [43]. To investigate the potential regulation of UCP-2 by the chromogranin A peptide PST, rat adipocytes were cultured in the same way as described for the study of leptin expression. Rat adipocytes were incubated in the absence or presence of 100 nM PST and the degree of UCP-2 expression was tested by RT-PCR. As shown in figure 2, PST stimulation for 16 h enhanced the expression of UCP-2 mRNA, about 100% over the control. This effect was already evident after 24 h incubation with a 50% increase over the control cells.

PST does not modify the expression of UCP-1 and PPAR-γ in rat adipocytes

Since UCP-1 has also been previously found to be present in white adipose tissue [31], and UCP-1 expression is upregulated by adrenergic activation [34], we tested the effect of PST on UCP-1 mRNA levels by RT-PCR. As shown in figure 3 A, UCP-1 is expressed in rat white adipose cells. However, stimulation of rat adipocytes with 100 nM PST for 16 and 24 h did not change the expression of UCP-1.

PPAR-γ mediates upregulation of UCPs [36–38, 44], and downregulation of leptin expression [45–47]. Since we have found that PST inhibits the expression of leptin and enhances the expression of UCP-2 in rat

adipocytes, we sought to check whether these effects of PST could be partly mediated by upregulation of PPAR-γ expression. As shown in figure 3, PST stimulation of rat adipocytes for 16 and 24 h did not affect the expression of PPAR-γ.

Inhibition of PKC blocks the effects of pancreastatin on leptin and UCP-2 expression

The metabolic effects of PST on rat adipocytes have been previously found to be mediated by PKC activation. Thus, we next studied the effect of PST on the expression of leptin and UCP-2 in the presence of a PKC inhibitor (50 nM bisindolylmaleimide). Adipocytes were preincubated with the PKC inhibitor for 5 min and then stimulated for 16 and 24 h in the presence or absence of PST (100 nM). As shown in figure 4 A, the effect of PST inhibiting the expression of leptin was completely reversed. Thus, no significant differences were found after 16- or 24-h incubation in the presence of PST, compared with controls. Furthermore, inhibition of PKC activity also completely blocked the inhibitory effect of PST on leptin secretion (fig. 4 B).

As shown in figure 5, the effect of PST enhancement of the expression level of UCP-2 was also completely blocked by PKC inhibition. Thus, no differences were found when the rat adipocytes were incubated in the presence of PST for 16 or 24 h, compared with control cells.

Discussion

The glycoprotein chromogranin A [48] is very abundant in chromaffin cells, but is also present throughout the neuroendocrine system [17]. The release of chromogranin A with catecholamines indicates that exocytosis is
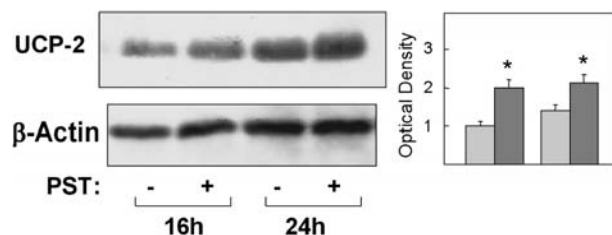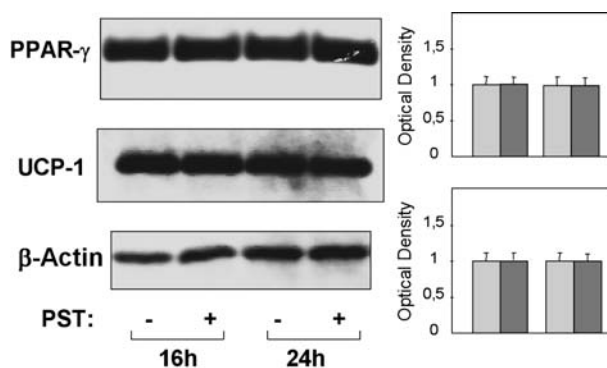

Figure 2. PST stimulates UCP-2 expression in rat adipocytes. Adipocytes were incubated in the absence or presence of 100 nM PST and UCP-2 mRNA expression was assessed by RT-PCR. UCP-2 bands were quantified and normalized with the β-actin bands. A representative experiment out of four is shown. Densitograms with SE are shown. * p < 0.05.

Figure 3. PST does not modify UCP-1 and PPAR-γ expression in rat adipocytes. Cells were incubated as described in figure 2 but UCP-1 and PPAR-γ primers were employed for RT-PCR. Total RNA was obtained and UCP-1 and PPR-γ mRNA was assessed by RT-PCR and Southern blot with an internal oligonucleotide. UCP-1 and PPAR-γ bands were quantified and normalized with the β-actin bands. An experiment representative of three is shown. Densitograms with SE are shown.
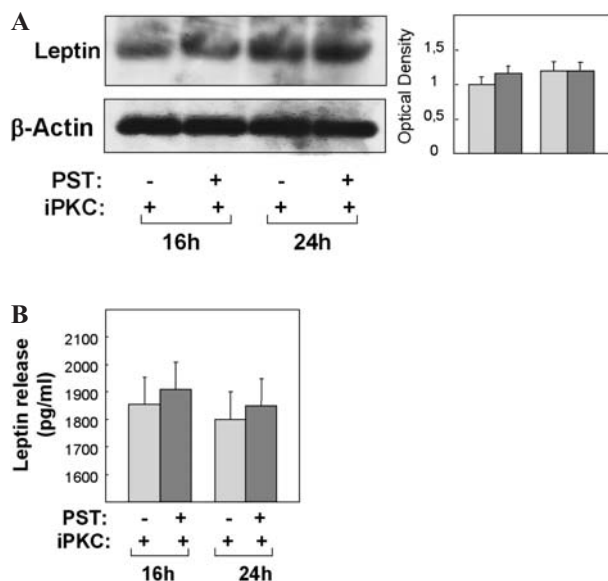
Figure 4. Inhibition of PKC activity blocks the effects of PST on leptin expression and secretion. Adipocytes were incubated as described in figure 1, but PKC activity was inhibited with 50 nM bisindolylmaleimide. (*A*) Total RNA was obtained and leptin expression was assessed by RT-PCR and Southern blot with an internal oligonucleotide. Leptin bands were quantified and normalized with the *β*-actin bands. A representative experiment out of three is shown. (*B*) Leptin levels were measured in the incubation medium by ELISA at 16 and 24 h. Data are means ± SE from three independent experiments run in duplicate.
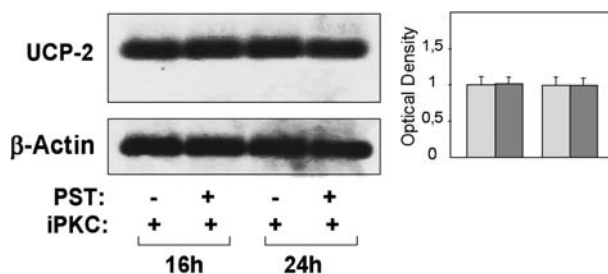

Figure 5. Inhibition of PKC activity blocks the PST stimulation of UCP-2 expression**.** Adipocytes were incubated as described in figure 2, but PKC activity was inhibited with 50 nM bisindolylmaleimide. UCP-2 mRNA expression was assessed by RT-PCR. UCP-2 bands were quantified and normalized with the *β*-actin bands. A representative experiment out of three is shown. Densitograms with SE are shown.

the mechanism of physiologic chromogranin release [49]. Thus, chromogranin A levels are a good indicator of sympathoadrenal activity [50], especially under stressful conditions, such as hypoglycemia [50, 51].

PST is well-established as one of the main bioactive peptides derived from chromogranin A [18, 19, 23]. The intracellular processing of PST involves endopeptidases, such as prohormone convertase-2 (PC-2) and carboxypeptidase H, whereas the extracellular processing of chromogranin A may occur either as a result of the activities of proteases co-secreted by the chromaffin granule,

to exoproteases localized on the extracellular side of the cell plasma membrane, or to the proteases present in plasma [52–54]. In any case, the sympathetic/adrenergic activity is the major source of chromogranin A in stressful conditions and is therefore also the major source for PST. In fact, PST has been shown to correlate with catecholamines in essential hypertension [21] and gestational diabetes [22], and a role for PST in the metabolic syndrome has been proposed [23, 55, 56].

Catecholamines are known to control the metabolic response to stress [57]. We have previously characterized the glycogenolytic and lipolytic effects of PST [22–28, 58], as well as counterregulatory effects on insulin action [59]. These effects are consistent with a putative role for PST potentiating the metabolic action of catecholamines [60].

We know that the adipocyte can centrally modulate adrenergic activity by producing leptin [61, 62]. In turn, adrenergic activation has been found to modulate leptin expression [15, 35]. The aim of the present work was to study the possible PST regulation of leptin expression and secretion, as well as the expression of other important genes for the energy balance of the adipocyte: UCP-1, UCP-2 and PPAR-*γ*. We found that PST differentially regulate the expression of these genes. Thus, PST inhibits leptin secretion from rat adipocytes by decreasing leptin mRNA expression and without modifying the release of synthesized leptin. This effect of PST on leptin is coincident with the inhibitory effect of adrenergic stimulation, and may contribute to the inhibition of leptin synthesis and secretion that occur upon sympathetic activation, since both PST and catecholamines are produced by chromaffin granules. The PST inhibition of leptin expression and secretion is transient and is no longer observed after 24 h. Since we employed static incubations, we do not know the possible contribution of the negative modulation of leptin secretion on its own expression, because leptin also has a well-known inhibitory effect on leptin expression by the adipocyte [63]. Thus, a decrease in leptin release may have the opposite effect, i. e. an increase in leptin mRNA expression. This autocrine effect of leptin may partly explain the transient inhibition of leptin expression by PST, which is no longer observed after 24 h.

The effect of PST inhibiting leptin production is counter to the previously reported effect of PST stimulating protein synthesis in the adipocyte [24, 64]. In fact, the PST inhibition of leptin release does not seem to be as dramatic as the inhibition of leptin mRNA expression. Therefore, this discrepancy may be explained by the dual effect of PST inhibiting leptin mRNA, but increasing protein synthesis. In any case, the final effect of PST is the transient inhibition of leptin release by the rat adipocyte. At the same time, PST stimulates the expression of UCP-2, the major uncoupling protein in white adipose tissue, without modifying the expression of UCP-1, which is

also present in white adipocytes. These results suggest that PST may work as an amplification of the adrenergic stimulation of basal metabolism increasing heat production, in a similar way to the reported effect increasing lipolysis [24]. This effect of PST on UCP-2 expression is still evident after 24 h, and therefore points to a role of PST regulating energy balance on a long-term basis. One should note that the effect of PST on UCP-2 expression may be even greater, since leptin is known to increase the expression of UCP-2 [63], and PST inhibits leptin secretion. On the other hand, PST, unlike catecholamines, has no effect on UCP-1 expression in rat white adipocytes. The lack of effect of PST on UCP-1 expression may reflect the different mechanisms of action exerted by PST and catecholamines. Nevertheless, we do not yet know the possible effect of PST on UCP-1 expression in its major localization, i.e. brown adipose tissue. This is an interesting hypothesis that may be worth investigation.

One of the known signals that mediates the increase in UCP-2 expression and inhibition of leptin is PPAR-γ. Thus, PPAR-γ activation has been shown to stimulate UCP-1 and UCP-2 expression [44], but to inhibit leptin expression [45–47]. That is why we checked the possible mediation of PPAR-γ on these PST effects. However, PST did not modify PPAR-γ expression, and therefore we may rule out an indirect effect of PST on leptin and UCP-2 expression via PPAR-γ expression. On the other hand, the production of activating PPAR-γ ligands by PST stimulation in rat adipocytes remains to be investigated.

The regulation of leptin and UCP-2 expression by PST is mediated by a PKC activation pathway, since both the inhibition of leptin and the increase in UCP-2 mRNA expression are prevented by blocking PKC activity. These results are consistent with the mechanism whereby PST exerts metabolic effects in the adipocyte, as well as the cross-talk with the insulin receptor [26–28]. Furthermore, previous work has already demonstrated that PKC activation negatively modulates leptin expression [65, 66], whereas these may be the first reported data regarding the role of PKC in UCP-2 expression.

Our current hypothesis is that PST plays a role in the physiology of stress, by regulating the supply of energy to the whole organism. In fact, PST levels may be increased under stressful conditions, during which chromogranin A is co-secreted with catecholamines. The present results further support the hypothesis that considers PST as a long-term effector of the adrenergic system by regulating the expression of important genes involved in the modulation of energy balance in white adipocytes.

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