Effects of dopamine on leptin release and leptin gene (OB) expression in adipocytes from obese and hypertensive patients

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Background: A reduction of dopaminergic (DAergic) activity with increased prolactin levels has been found in obese and hypertensive patients, suggesting its involvement as a pathophysiological mechanism promoting hypertension. Similarly, leptin action increasing sympathetic activity has been proposed to be involved in mechanisms of hypertension. The aim of this study was to analyze the effects of DA, norepinephrine (NE), and prolactin on leptin release and leptin gene (OB) expression in adipocytes from obese and hypertensive patients.

Methods: Leptin release and OB gene expression were analyzed in cultured adipocytes from 16 obese and hypertensive patients treated with DA (0.001, 0.01, 0.1, and 1.0 µmol/L), NE (1.0 µmol/L), insulin (0.1 µmol/L), and prolactin (1.0 µmol/L), and from five nonobese and normotensive controls treated with DA (1 µmol/L), NE (1 µmol/L), insulin (0.1 µmol/L), and prolactin (1.0 µmol/L).

Results: A dose-related reduction of leptin release and OB gene messenger ribonucleic acid expression under different doses of DA was observed in adipocytes from obese hypertensive patients. Whereas prolactin treatment elicited a significant increase of both leptin release and OB gene expression, NE reduced these parameters. Although similar effects of DA and NE were observed in adipocytes from controls, baseline values in controls were reduced to 20% of the value in adipocytes from obese hypertensive patients.

Conclusion: These results suggest that DAergic deficiency contributes to metabolic disorders linked to hyperleptinemia in obese and hypertensive patients.

Keywords: dopamine, leptin, cultured adipocytes, obesity, hypertension

Introduction
The association between hypertension, obesity, and insulin resistance1 is closely linked to increased sympathetic activity.2 The dopaminergic (DAergic) influence on blood pressure regulation, including DAergic-mediated inhibition of norepinephrine (NE) at sympathetic neuroeffector endings, leads to a reduction of central and peripheral DA activity and a hypertensive condition, possibly accounting for essential hypertension.3 Clinical studies show a DAergic modulation of sympathetic activity in hypertensive and obese individuals,4 and DAergic agonists have been successfully used as a treatment for hypertension by reducing adrenergic neurotransmission at sympathetic endings.5 As bromocriptine treatment in obese humans elicited a reduction of serum leptin values6 and improved insulin sensitivity,7 this indirectly suggests beneficial effects of reduced DAergic activity.

Leptin, the peptide product of the obesity gene (OB) expressed and released mainly from adipocytes,8 plays an important role in the regulation of food intake, energy...
expenditure, and body weight regulation. High plasma levels of leptin in obese subjects reflect leptin resistance, which may contribute to obesity-related hypertension, ie, chronic leptin infusion increases sympathetic activity and arterial pressure. Whereas several endogenous compounds, including insulin, glucocorticoids, and prolactin, may increase OB gene expression from adipocytes, NE induces the opposite effect. Hence, a leptin-mediated pathophysiological influence of these endogenous compounds has been suggested in obesity and hypertension.

The aim of this study was to explore if DAergic deficiency could contribute at least in part to metabolic disorders, particularly hyperleptinemia in obese and hypertensive patients. For this purpose, we examined the effects of DA, NE, insulin, and prolactin on leptin release and OB gene expression in adipocytes from obese hypertensive patients and age- and sex-matched nonobese normotensive patients undergoing elective surgery.

**Materials and methods**

**Study design**

The study comprised 16 obese hypertensive patients (ten women and six men, body mass index [BMI] ≥30 kg/m²) and five nonobese normotensive patients (three women and two men, BMI <30 kg/m²) serving as controls who underwent elective surgery for cholecystitis (n=7), umbilical hernia (n=12), lipoma (n=1), and polycystic ovary (n=1). The hypertensive patients had on at least two occasions during a 1-week interval systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg, and mean blood pressure ≥107 mmHg (after a 5-minute rest period and seated). No subjects had renal, hepatic, or cardiac disease. Six subjects were taking antihypertensive drugs: angiotensin-converting enzyme (ACE) inhibitors (n=4), calcium antagonists (n=1), and ACE-inhibitors plus diuretic (n=1) at the time of surgery.

The local research committee of the General Regional Hospital Number 1, Instituto Mexicano del Seguro Social (IMSS), Morelia, Michoacán, México, approved the experimental protocol. All patients gave informed consent to participate in the study.

**Samples**

Abdominal subcutaneous adipose tissue samples weighing from 2.5 to 10 g were obtained by biopsy during elective abdominal surgery, under general anesthesia or epidural blockage, 5 minutes after opening the abdominal wall and before further surgical procedures. The patients had fasted from 10 pm the previous day before undergoing surgery. General anesthesia was induced at about 8 am by a short-acting barbiturate and maintained by sevoflurane plus fentanyl and oxygen (balanced anesthesia) or epidural blockage induced with lidocaine 2%. After removal of adipose tissue, specimens were stored in sterile cold (2°C–4°C) phosphate-buffered saline (PBS) with the addition of 500 IU/mL penicillin and 500 µg/mL streptomycin and immediately transferred to the laboratory to be processed within the following 30 minutes.

**Materials**

DA, NE, prolactin, and propranolol (β-adrenergic receptor antagonist) were purchased from Sigma-Aldrich (St Louis, MO, USA). Metoclopramide (DAergic antagonist) and NPH human insulin were provided by the pharmaceutical companies Hoechst Marion Russell (Mexico City, Mexico) and Eli Lilly (Mexico City, Mexico), respectively. Dulbecco’s Modified Eagle’s Medium (DMEM), bovine serum albumin (BSA), collagenase type II, PBS, penicillin and streptomycin, and cell-culture reagents were purchased from Life Technologies (Carlsbad, CA, USA). A Titan one-tube reverse-transcription polymerase chain reaction (RT-PCR) kit (Roche Diagnostics, Basel, Switzerland) was used RT and PCR. Oligonucleotides were purchased from Life Technologies.

**Cell culture**

The isolation and culture of the adipose tissue cells were performed following the Rodbell procedure, with minor modifications, as follows. Tissue samples were weighed and minced; tissue fragments were then digested in fresh PBS penicillin/streptomycin buffer in the presence of type II collagenase (2 mg/mL,buffering tissue) at 37°C with gentle shaking at 200 cycles/minute for 45 minutes. The resulting cell suspension was separated from undigested tissue by filtration through a 400-µM nylon mesh and washed three times. For each wash, cells were centrifuged at 500 rpm for 1 minute, and the infranatant was discarded each time. The supernatant adipocytes were resuspended in PBS penicillin/streptomycin buffer. The final wash was in DMEM supplemented with 10% BSA, sodium bicarbonate, HEPES (4-[2-hydroxyethyl]-1-piperazinethanesulfonic acid), penicillin/streptomycin (penicillin 500 IU/mL and streptomycin 500 µg/mL). It was then incubated for 30 minutes at a constant temperature of 37°C and 5% CO₂, before being plated in culture plates in order to continue the incubation for 72 hours, with DMEM replacement at 24-hour intervals. Viability of the adipocytes was microscopically monitored (ID03 phase-contrast microscope;
Zeiss, Jena, Germany). Then, the DMEM, without BSA, was used for maintenance of cultured adipocytes during the following treatments: DA (0.001, 0.01, 0.1, and 1.0 µmol/L); DA (1.0 µmol/L) plus metoclopramide (1.0 µmol/L); NE (1.0 µmol/l); NE (1.0 µmol/L) plus propranolol (20 µg/mL); and insulin (0.1 µmol/L) or prolactin (1.0 µmol/L). All treatments were maintained for 24 hours, with the exception of insulin treatment (which was maintained for 72 hours), in order to evaluate their effects on adipocyte OB gene expression and leptin release. Each experiment was performed six times in different cultured adipocytes obtained by biopsies, ie, in six plates incubated simultaneously, and was repeated in two separate experiments. At the end of the 24- or 72-hour treatment period, culture medium corresponding to adipocytes receiving the different treatments was frozen and stored at −70°C until assay. Cultured adipocytes in the plates under the different treatments were prepared for total ribonucleic acid (RNA) extraction.

Total RNA extraction and reverse-transcription polymerase chain reaction
Total RNA from cultured cells was prepared with the use of TRIzol® Reagent (Life Technologies) according to the manufacturer’s protocol. Agarose gel electrophoresis and ethidium bromide staining assessed the integrity of the adipocyte RNA samples. The RNA samples were then diluted appropriately to equalize concentrations, and stored at −70°C until use. RT and amplification of complementary deoxyribonucleic acid (cDNA) was carried out by PCR. RT-PCR was performed with the Titan one-tube RT-PCR kit. Using avian myeloblastosis virus for first-strand synthesis and the Expand High-Fidelity enzyme blend (Roche), which consisted of Taq DNA polymerase and Pwo DNA polymerase for the PCR part, a one-step reaction system was performed. In addition, the system included a single optimized RT-PCR buffer, control RNA from a human cell line (K562), and control primers for human β-actin messenger RNA (mRNA). Master mix 1 was composed of deoxyribonucleotide triphosphate mix 0.2 nM (each), dithiothreitol solution 5 mM, ribonuclease inhibitor 5 U, control primer mix 0.4 µM (each), downstream leptin primer 0.4 µM (each), upstream leptin primer 0.4 µM, control RNA 10 pg, template RNA 600 ng, and sterile water for a volume of 25 µL. Master mix 2 was composed of 5× RT-PCR buffer, enzyme mix (1 µL), and sterile water for a volume of 25 µL. Then, 25 µL each of master mixes 1 and 2 was added to a 0.2 mL thin-walled PCR tube on ice. The sample was mixed and collected at the bottom of the tube, and was briefly centrifuged and overloaded with 30 µL mineral oil.

The PCR primers used were: leptin, sense 5′-CAT TGG GGA ACC CTG TGC GGA-3′ and antisense 5′-TGG CAG CTC TTA GAG AAG GCC-3′; β-actin, sense 5′-CCA AGG CCA ACC GCG AGA AGATGA C-3′ and antisense 5′-AGG GTA CAT GGT GGT GCC AGA C-3′. PCR products of leptin and β-actin generated fragments of 260 bp and 587 bp, respectively, when they were amplified. The β-actin was used as the housekeeping gene and amplified with leptin as internal control. Samples were placed in a Delta Cycler System P/W TCX80 (San Diego, CA, USA), equilibrated at 50°C, and incubated for 30 minutes for the RT. The cDNA synthesis reaction PCR was accomplished in 37 cycles; 1 × denature template at 94°C for 1 minute, 10 × denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 2 minutes; 25 × denaturation at 94°C for 1 minute, annealing at 60°C for 30 seconds, and elongation at 72°C for 2 minutes; elongation at 72°C (2 minutes) plus five seconds for each cycle (eg, cycle 11 added 5 seconds, cycle 12 added 10 seconds, cycle 13 added 15 seconds); 1x of elongation time at 72°C lasted 7 minutes. The products of the amplification were resolved by electrophoresis in 2% agarose gel. The band densities were quantified using the RFLPscan 2.1 software (Scan analytics, Billerica, MA, USA).

Measurement of leptin
Leptin levels in the culture medium were measured by RIA using a human leptin RIA kit (Linco Research, St Charles, MO, USA). The limit of sensitivity was 0.05 ng/mL. The intra- and interassay coefficients of variation for concentrations of 0.58 ng/mL and 0.53 ng/mL were 1.8% and 4.9% and 3.6% and 6.6%, respectively.

Statistical analysis
Results from six experiments are expressed as means ± standard error. Statistical tests were performed using the SPSS, version 18 for Windows (IBM, Armonk, NY, USA). The Kolmogorov–Smirnov one-sample test was used to test for normal distribution of data. Student’s t-test was used for comparisons of the means of the values of the clinic variables and leptin release of the cultured adipocytes from obese hypertensive and nonobese normotensive patients. One-way analysis of variance followed by Tukey’s post hoc test was used to compare mean values of leptin release and OB gene expression under different treatments, with P<0.05 considered statistically significant.
Results
The clinical characteristics of the subjects are shown in Table 1. At baseline, significant differences between the groups were found in body weight, BMI, and systolic, diastolic, and mean blood pressure.

Figure 1 illustrates changes in leptin release elicited by the different treatments on cultured adipocytes. A significant reduction of leptin was observed when adipocytes were under DA, NE, and NE plus propranolol treatments, whereas there was a significant increase of leptin release under prolactin treatment compared to leptin release in untreated adipocytes. The changes observed in values of leptin in culture medium were not significantly different under DA plus metoclopramide, or insulin treatments compared to the control. The data for leptin release in the two groups are depicted in Figure 2. Leptin release from cultured adipocytes from obese hypertensive patients was fivefold higher than cultured adipocytes from the nonobese normotensive patients (P<0.0001) under control conditions. Figure 3A shows a significant reduction in leptin release obtained in cultured adipocytes from obese hypertensive patients exposed to 1 µmol/L DA (by 0.45±0.325 ng/mL, P=0.04) and 1.0 µmol/L NE (by 0.300±0.226 ng/mL, P=0.001) and a significant increase with 1.0 µmol/L prolactin (by 0.862±0.366 ng/mL, P=0.04), whereas only a significant reduction in leptin release was observed in cultured adipocytes from nonobese normotensive patients exposed to 1.0 µmol/L DA (by 0.062±0.016 ng/mL, P=0.008) and 1.0 µmol/L NE (by 0.054±0.016 ng/mL, P=0.005). These results are shown in Figure 3B. Insulin (0.1 µmol/L) and prolactin (1.0 µmol/L) treatments were ineffective.

Figure 4 illustrates percentage changes in the content of OB gene mRNA expression of cultured adipocytes under the different treatments in comparison to leptin mRNA band density in cultured adipocytes not exposed to any treatment (100%). A significant reduction of OB gene mRNA expression was observed under 1.0 µmol/L of DA (to 54%±4%, P=0.02), while the effect under DA plus metoclopramide treatment was not statistically significant. In addition, NE elicited a significant reduction of OB gene mRNA expression (to 68%±6%, P=0.001), which was not completely reversed by propranolol. Insulin treatment did not induce significant modifications in OB gene mRNA expression of cultured adipocytes. Nevertheless, prolactin treatment elicited a significant increase of OB gene mRNA expression (to 48%±18%, P=0.04).

In addition, when cultured adipocytes of obese hypertensive patients were exposed to different concentrations of DA, a dose-dependent reduction of OB gene mRNA expression was observed, with significant reductions attained using DA 0.1 µmol/L and DA 1.0 µmol/L (52%±2.4%, P<0.05, and 63%±1.2%, P=0.01, respectively), and leptin release (from 0.71±0.329 ng/mL to 0.47±0.116 ng/mL, P=0.01, and from 0.71±0.329 ng/mL to 0.32±0.08 ng/mL, P<0.0001, respectively) (Figure 5A–C).

Discussion
Our data confirm that leptin release was fivefold higher in cultured adipocytes obtained from obese hypertensive patients compared to nonobese normotensive patients. Moreover, this study demonstrates that whereas DA and NE exert inhibitory effects on leptin release and OB gene expression, prolactin increases leptin release and OB gene expression in cultured adipocytes from both obese hypertensive patients and nonobese normotensive patients. Thus, our results suggest that DAergic deficiency may contribute at least in part to metabolic disorders linked to hyperleptinemia in obese hypertensive patients.

The finding that NE reduced OB gene expression is in agreement with results from previous studies in cultured 3T3-L1 adipocytes16 and in cultured adipocytes from obese patients.17 Moreover, NE addition to the culture resulted in a significant reduction of leptin release from cultured adipocytes from obese hypertensive patients, which was partially prevented by propranolol administration, in agreement with a previous report.16 The partial reduction of the NE effects induced by propranolol could be due to the participation of

Table 1 Clinical characteristics of obese hypertensive patients and nonobese normotensive patients who provided adipose tissue samples for cultured adipocytes

|                     | Obese and hypertensive patients* | Nonobese and normotensive patients* | P     |
|---------------------|---------------------------------|------------------------------------|-------|
| Age (years)         | 51±8                            | 48±4                               | 0.424 |
| Sex (male/female)   | 6/10                            | 2/3                                |       |
| Weight (kg)         | 83±8                            | 63±6                               | 0.005 |
| Height (m)          | 1.59±0.5                        | 1.59±0.4                           | 0.798 |
| BMI (kg/m²)         | 32.9±2.0                        | 24.9±1.1                           | <0.0001|
| Systolic blood pressure (mmHg) | 149±9                      | 120±7                              | <0.0001|
| Diastolic blood pressure (mmHg) | 93±5                        | 78±4                               | <0.0001|
| MBP (mmHg)**        | 111±5                           | 92±5                               | <0.0001|

Notes: *Mean ± standard error; **MBP = diastolic pressure + 1/3 of difference (systolic/diastolic pressure).

Abbreviations: BMI, body mass index; MBP, mean blood pressure.
Dopamine effect on leptin release and gene expression

Figure 1: Effects of different treatments on leptin release in cultured human adipocytes from obese hypertensive patients. Values (means ± standard error) are expressed as leptin concentrations in the culture medium. Control (CO), regular medium alone; dopamine (DA; 1.0 µmol/L); DA (1.0 µmol/L) plus metoclopramide (MET; 1.0 µmol/L); norepinephrine (NE; 1.0 µmol/L); NE (1.0 µmol/L) plus propranolol (PROPR; 20 µg/mL); insulin (INSUL; 0.1 µmol/L); and prolactin (PRL; 1.0 µmol/L).

Notes: *P<0.05 versus control (as the reference group). One-way analysis of variance and Tukey’s post hoc test, n=7 per group, performed in duplicate.

The β₂-adrenergic receptor, because propranolol is known to be an antagonist for the β₁- and β₂-adrenergic receptors. Thus, our results support the hypothesis of noradrenergic involvement in cellular mechanisms resulting in leptin release and OB gene expression, suggesting that leptin release is modified in parallel with OB gene expression.

The chief finding of the present study was the DAergic effect on leptin release and OB gene expression in cultured adipocytes, similar to that induced by NE. In fact, DA elicited a dose-related reduction of both leptin release and OB gene expression in cultured adipocytes, possibly mediated by DA₂ receptors, since metoclopramide administration resulted in

Figure 2: Values (means ± standard error) of leptin release from cultured adipocytes from non-obese normotensive patients, and from cultured adipocytes from obese hypertensive patients. Note the significantly higher values of leptin release from adipocytes from obese hypertensive patients in both control and treatment conditions.

Notes: *P<0.05, Student’s t-test; control (CO), regular medium alone; dopamine (DA; 1.0 µmol/L); norepinephrine (NE; 1.0 µmol/L); insulin (INSUL; 0.1 µmol/L); and prolactin (PRL; 1.0 µmol/L).
abolishment of the DA effect. Involvement of G (G_i/G_o) proteins, adenylate cyclase, and phospholipase C has been proposed, accounting for DA_2-mediated DA effects. Since DA and NE have negative effects on leptin release and OB gene expression, it appears that increments in body fat and as a consequence leptin overproduction precede sympathetic activity. This finding suggests that interactions between sympathetic overactivity and hyperleptinemia in obese hypertensive patients are dependent primarily on increments in body fat.
Although we did not test the effect of leptin on DA or NE production, circulating leptin has been suggested to contribute to increased blood pressure in obesity by activation of the sympathetic nervous system. As leptin-binding sites have been found in regions of the brain that are important in cardiovascular control and chronic leptin infusion exerts long-term effects on cardiovascular and renal function, it seems conceivable that leptin could modify cardiovascular function through central nervous system effects. Evidence suggests that endogenous DA modulates NE synthesis and secretion in the noradrenergic neurons and – at least in part – leptin release and OB gene expression in adipose tissue. As DAergic activity is reduced in subjects with hypertension and obesity, this could explain in part that obesity, sympathetic hyperactivity, and hyperleptinemia are associated with hypertension. We did not find effects of insulin on leptin release and OB gene expression in cultured adipocytes. These results are in accordance with previous reports published on primary cultures of adipocytes. However, other studies have demonstrated that insulin causes an increase in leptin release.
and OB gene expression both in vivo\textsuperscript{39} and in vitro.\textsuperscript{13} There are at least three possible explanations for this discrepancy. One is the type of insulin used. In this study, NPH human insulin was used with pharmacokinetic properties of onset of action of 1–2 hours, maximum action of 6–12 hours, and duration up to 18–24 hours. However, we measured leptin at 24, 48, and 72 hours, so the effect might have been able to be observed before 24 hours. Another explanation is that cultured cells require a longer stimulation period; however, in the present study, this is not a probable explanation, since we kept the cells in culture for 72 hours. A third explanation may be that insulin causes an increase in leptin release and OB gene expression only in vivo.

Although prolactin is a peptide hormone produced mainly in the anterior pituitary, it is also synthesized in extrapituitary sites,\textsuperscript{30} including adipocytes. Hyperprolactinemia, suggesting decrease of DAergic activity, is found in patients with hypertension,\textsuperscript{31} obesity,\textsuperscript{32} and chronic kidney disease,\textsuperscript{33} ie, conditions associated with hyperleptinemia. We confirm that prolactin increases leptin release and OB gene expression in adipocytes cultured from subcutaneous fat of humans. However, results of recent studies suggest that the response is reciprocal in both directions, since on the one hand it has been found that leptin regulates DA in response to sustained stress and eating behavior in humans, implicating involvement of DAergic pathways in this response,\textsuperscript{34,35} and on the other hand a study in an experimental model showed that prolactin inhibits lipolysis and leptin release.\textsuperscript{36} These discrepancies may be due to several factors, such as doses of DA and prolactin, experimental design, species, or sex.

Figure 5 (A–C) Dose-dependent reduction of OB gene messenger ribonucleic acid (mRNA) and leptin release induced by dopamine (DA) in cultured human adipocytes of obese, hypertensive patients. (A) Representative bands of OB gene mRNA. (B) Percentage values (means ± standard error) of leptin mRNA in reference to control conditions. (C) Leptin release under different concentrations of DA in reference to control. Notes: *P<0.05, one-way analysis of variance and Tukey’s post hoc test, n=5 per group, performed in duplicate.
Summary and conclusion

In the current study, DA and NE were found to decrease leptin release and OB gene expression in cultured adipocytes from subcutaneous fat tissue of obese hypertensive patients and nonobese normotensive patients, while there was an opposite effect of prolactin in cultured adipocytes from obese hypertensive patients. The clinical significance of these findings remains to be clarified in future studies; however, our observations support links between hyperprolactinemia, fat mass, hyperleptinemia, and hypertension. While recognizing that the investigated interactions are much more complex in vivo in the setting of numerous interrelated pathways interacting simultaneously, and likely to result in a variable response, the in vitro study of the dose-dependent response of these pathways under controlled situations, such as in the current study, could be a useful step to further our understanding of the much more complex mechanism in vivo in a sequential manner.

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Disclosure

The authors report no conflicts of interest in this work.

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