Dopamine D2 receptor upregulates leptin and IL-6 in adipocytes

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Abstract  Leptin is a pro-inflammatory cytokine secreted by the adipose tissue. Dopamine D2 receptors (D2Rs) have anti-inflammatory effects in the brain and kidney tissues. Mouse and human adipocytes express D2R; D2R protein was 10-fold greater in adipocytes from human visceral tissue than subcutaneous tissue. However, the function of D2R in adipocytes is not well understood. 3T3-L1 cells were treated with D2-like receptor agonist quinpirole, and immunoblot and quantitative PCR were performed. Quinpirole increased the protein and mRNA expression of leptin and IL-6, but not adiponectin and visfatin (24 h). It also increased the mRNA expression of TNF-α, MCP1, and NFκB-p50. An acute increase in the protein expression of leptin and TNF-α was also found in the cells treated with quinpirole. The leptin concentration in the culture media was increased by quinpirole-bathing the 3T3-L1 adipocytes. These quinpirole effects on leptin and IL-6 expression were prevented by the D2R antagonist L741,626. Similarly, siRNA-mediated silencing of Drd2 decreased the leptin, IL-6, mRNA, and protein expressions. The D2R-mediated increase in leptin expression was prevented by the phosphoinositide 3-kinase inhibitor LY294002. Acute quinpirole treatment in C57Bl/6J mice increased serum leptin concentration and leptin mRNA in visceral adipocyte tissue but not in subcutaneous adipocytes, confirming the stimulatory effect of D2R on leptin in vivo. Our results suggest that the stimulation of D2R increases leptin production and may have a tissue-specific pro-inflammatory effect in adipocytes.—Wang, X., V. A. Villar, A. Tiu, K. K. Upadhyay, and S. Cuevas. Dopamine D2 receptor upregulates leptin and IL-6 in adipocytes. J. Lipid Res. 2018, 59: 607–614.

Supplementary key words dopamine receptors • inflammation • adipocyte

Adipose tissue is involved not only in the storage and mobilization of lipids but also acts as an endocrine organ by producing numerous cytokines, such as pro-inflammatory molecules. These pro-inflammatory molecules include interleukin (IL)-6, TNF-α, and adipokines such as leptin. Leptin regulates food intake, energy homeostasis, and the production of other pro-inflammatory cytokines, such as IL-6, as well as anti-inflammatory cytokines (3, 4). IL-6, expressed in preadipocytes and adipocytes, causes a mutually inductive, positive “feedback loop” of inflammation in adipose tissue (5). Adipokines are known markers of inflammation that are associated with cardiovascular risk factors (1). Obesity, which is a risk factor for cardiovascular disease, is associated with elevated serum levels of several pro-inflammatory markers (6). Inflammation may be an important link between obesity and cardiovascular disease (1).

Dopamine receptors, which belong to the G protein-coupled receptor family, are classified into two families: D1-like (D1R and D5R) and D2-like (D2R, D3R, and D4R) dopamine receptors. Dopamine is well known as a neurotransmitter. However, dopamine can regulate other cellular functions, including ion transport, smooth muscle contractility, hormone production, and cell proliferation (7–9). Dopamine can also regulate inflammation. Mouse and human adipocytes express dopamine receptors but their effects on inflammation are controversial. Thus, dopamine has been reported to decrease (10–12) leptin expression and increase IL-6 expression in adipocytes (10, 13). D1-like receptor stimulation decreased leptin release but increased IL-6 release in subcutaneous adipocytes from nonobese, non-diabetic humans (10). However, the role of the dopamine D2 receptor (D2R) on adipokine expression in adipocytes is not known. Therefore, we tested the hypothesis that the
D_{2R} regulates the expression of leptin and IL-6 in a mouse adipocyte cell line.

**MATERIALS AND METHODS**

**Human subcutaneous and visceral adipocyte cells**

Preadipocytes were isolated from subcutaneous and visceral fat of nonoverweight, nondiabetic donors undergoing elective abdominal surgery. Preadipocytes were cultured and then differentiated into adipocytes, using differentiation medium as described previously (14).

**3T3-L1 cell culture**

Mouse 3T3-L1 cells were obtained from Zen-Bio, Inc. The maintenance and differentiation of 3T3-L1 preadipocytes from adipocytes was carried out following the protocol provided by the company (Zen-Bio, Inc.). 3T3-L1 preadipocyte medium (PM-L1), 3T3-L1 differentiation medium (DM-2-L1), and 3T3-L1 adipocyte medium (AM-L1-IF) were from Zen-Bio, Inc.

Differentiated 3T3-L1 adipocyte cells were serum-starved for 2 h and treated for 24 h with 1 µM quinpirole (D_{2R}/D_{3R} agonist, Sigma-Aldrich), or 1 µM quinpirole plus 1 µM L-741,262 (selective D_{3R} antagonist, Sigma-Aldrich), as previously described (15, 16). Differentiated 3T3-L1 adipocytes were transfected with non-silencing siRNA (30 nM, Qiagen) or D2d2 siRNA (30 nM, Qiagen) using Hyperfect (Qiagen) and studied 72 h posttransfection. In additional experiments, 3T3-L1 adipocytes were treated with vehicle, quinpirole (1µM; 24 h), or dopamine (1µM; 24 h) in the presence or absence of SCH 23390 (D_{1}-like receptor antagonist, 10 µM), L741,262 (10 µM), propranolol (β-adrenergic receptor antagonist, 10 µM), phenolamine (α-adrenergic antagonist, 10 µM), and LY294002 (PI 3K inhibitor, 10 µM), or L39A/D40A/F41 (leptin antagonist, 25 nM, 24 h) (17). 3T3-L1 adipocytes were also treated with quinpirole and dopamine, with or without insulin. The 3T3-L1 adipocyte medium without insulin (AM-L1-IF) or one that was supplemented with insulin (1µg/ml) were used.

**Mice**

C57Bl/6J male mice at 20 weeks of age (Jackson Laboratory, Bar Harbor, ME) were housed in the Animal Care Facility of the University of Maryland Baltimore and were studied for the role of D_{2R} on the expression and release of leptin in adipocytes in vivo. The mice were treated with quinpirole (1 mg/kg; i.v.) or vehicle; the serum samples and adipocyte tissue (subcutaneous and visceral) were harvested 2 h after injection. All studies were approved by the Animal Care and Use Committee of the University of Maryland Baltimore.

**Immunoblotting**

Mouse kidney homogenates and cell lysates were subjected to immunoblotting as previously described (15, 16). The primary antibodies used were rabbit polyclonal anti-D_{2R} (Millipore, #AB5084P), rabbit polyclonal antibodies against leptin (ABCAM, #ab9749), IL-6 (ABCAM, #ab6672), adiponectin (ABCAM, #ab22554), visfatin (BioVision, anti-mouse #5908-100), TNFα (ABCAM, #ab9739), OB receptor (Gene-Tex, Inc. GTX25593), and mouse monoclonal anti-GAPDH (Millipore,#MAB374). The densitometry values were corrected by the expression of GAPDH.

**Quantitative real-time PCR**

Total RNA was purified using the RNeasy RNA extraction Mini kit (Qiagen, Valencia, CA). RNA samples were converted into first strand cDNA using an RT2 First Strand kit (Qiagen). Gene expression was quantified by real-time PCR performed on an ABI Prism 7900 HT (Applied Biosystems, Foster City, CA). The assay used gene-specific primers (Qiagen) and the SYBR Green real-time PCR detection method (Qiagen). GAPDH was used as a housekeeping gene. The different genes studied were those for leptin, IL-6, TNFα, MCP-1, NFκB p50, and GAPDH. Data were analyzed using the ∆ΔCt method (18).

**Leptin concentration in the medium**

Leptin concentration in cell culture medium was quantified using a commercial Kit (Cell Biolabs, INC). All assays were performed in duplicate and normalized by protein concentration.

**Statistical analysis**

Data are expressed as mean ± SEM. Comparisons between two groups used the Student’s t test. One-way ANOVA followed by posthoc analysis using the Holm-Sidak multiple comparison test were used to assess significant differences among three or more groups. P < 0.05 was considered statistically significant.

**RESULTS**

D_{2R} and D_{3R} are expressed higher in human visceral than subcutaneous adipocytes

The presence of D2R and D3R in primary cultures of human subcutaneous adipocytes and in mouse 3T3-L1 cells was confirmed by immunoblotting (Fig. 1A, B). To compare the relative abundance of D_{2R} in visceral and subcutaneous adipocytes, primary cultures of human adipocytes from subcutaneous and visceral fat were studied. qRt-PCR analyses showed that D_{2R} mRNA was 10-fold greater in visceral than subcutaneous adipocytes (Fig. 1C).

[Fig. 1. D_{2R} and D_{3R} are expressed in human adipocytes and mouse adipocytes. A, B: The expression of D_{2R} and D_{3R} was determined by Western blot in human subcutaneous adipocytes cells and 3T3-L1 cells. Mouse proximal tubular cells were used as a positive control (PC). PA, preadipocytes; A, adipocytes. C: D_{2R} and D_{3R} mRNA expression was determined by qRT-PCR in human preadipocytes and adipocytes. The data were normalized with GAPDH.]
**D2R stimulation increases leptin and IL-6 expression in 3T3-L1 cells**

Stimulation of the D2R with quinpirole (1 μM, 24 h) had no effect on protein expression of adiponectin and visfatin, but increased the protein expression of leptin (+23 ± 8%, n = 4, P < 0.05) and IL-6 (+49 ± 7%, n = 4, P < 0.05). These effects were prevented by a D2R-selective antagonist (L741,626, 1 μM, 24 h) (Fig. 2A), which did not have effects on leptin and IL-6 expression by itself. Leptin and TNFα increased with quinpirole stimulation for 15 min (leptin +94 ± 7%, P < 0.05), 30 min (leptin +60 ± 14%, n = 4, P < 0.05) and 2 h (leptin +60 ± 14%, n = 4, P < 0.05) (Fig. 2B). D2R stimulation after 24 h also increased mRNA expression of leptin (1.50 ± 0.03-fold, n = 5, P < 0.05), IL-6 (2.44 ± 0.08-fold, n = 5, P < 0.05), TNFα (2.23 ± 0.06-fold, P < 0.05), MCP1 (1.82 ± 0.04-fold, P < 0.05), and NFκB p50 (1.47 ± 0.02-fold, P < 0.05) (Fig. 2C). Leptin concentration in the medium also increased after a 24 h quinpirole treatment (1 μM) (+78 ± 19%, n = 4, P < 0.05) (Fig. 2D). These data show that D2R upregulates the protein expression of adipokines, mainly leptin, IL-6, and possibly TNFα, in 3T3-L1 cells by increasing their mRNA expression.

**Drd2 silencing decreases leptin and IL-6 expression in mouse 3T3-L1 cells**

Silencing D2R expression with Drd2 siRNA decreased protein expression of D2R (−51.7 ± 9.6%, n = 4, P < 0.05), leptin (−51.7 ± 9.6%, n = 4, P < 0.05), and IL-6 (−24.7 ± 5.2%, n = 4, P < 0.05) (Fig. 3A), and leptin level in the media (−26.2 ± 3.9% pg/mg protein, P < 0.05) (Fig. 3B). The protein expression of D2R and Drd2 was not altered demonstrating the specificity of the siRNA (Fig. 3A). The mRNA expression of leptin (−0.38 ± 0.01-fold, n = 5, P < 0.05), and IL-6 (0.58 ± 0.01-fold, n = 5, P < 0.05) were also decreased (Fig. 3C) after silencing the Drd2 in 3T3-L1 cells. This is in contrast to the ability of D2R to inhibit the expression of pro-inflammatory cytokines and chemokines in renal proximal tubule cells (16). We suggest that the effect of D2R on cytokines and chemokines is tissue-specific.

**D2R regulates leptin expression via AKT in 3T3-L1 cells**

Stimulation of D2R by quinpirole (1 μM; 24 h) increased phospho AKT and leptin protein expression (+131 ± 36%, P < 0.05, n = 4) (Fig. 4). This is in agreement with the data shown in Fig. 2 where the effects were completely prevented by cotreatment with the reversible PI3K inhibitor LY294002 (10 μM; 24 h), suggesting that AKT may be involved in the positive regulation of leptin by D2R. By contrast, the D2R-stimulated increase in IL-6 protein expression (+120 ± 24%, n = 4, P < 0.05) was not prevented completely by LY294002 (+71 ± 23%, n = 4, P < 0.05) (Fig. 4A). We speculate that PI3K regulates the de novo synthesis of leptin but not its release. Therefore, the presence of the residual leptin in the medium may have kept the IL-6 expression high even in the presence of PI3K inhibitor. To determine whether the presumed residual effect of leptin on IL-6 expression is correct, differentiated 3T3-L1 cells were treated...
with L39A/D40A/F41 (25 nM, 24 h), a leptin antagonist (17), and quinpirole (1 μM, 24 h). Immunoblot analyses show that IL-6 protein expression was decreased in the presence of L39A/D40A/F41, and quinpirole did not increase IL-6 protein expression in the presence of the leptin antagonist (−31 ± 6%, n = 5, P < 0.05) (Fig. 4C), suggesting that the presence of leptin in the medium may have been responsible for the inability of the PI3K inhibitor (LY294002) to block the D2R stimulatory effect on IL-6 expression (Fig. 4A). The presence of leptin receptor (OB-R) in 3T3-L1 cells was confirmed by immunoblotting. Its expression was not altered by D2R stimulation (Fig. 4B).

Insulin modulates the stimulatory effect of D2R on leptin expression in 3T3-L1 cells

Insulin is one of the most important stimuli of leptin expression and secretion via the PI3K pathway (19–22). Our data show that D2R also stimulates leptin expression and secretion in 3T3-L1 cells, via the PI3K/AKT pathway. We next determined whether insulin plays a role in the D2R-mediated stimulation of leptin and IL-6. According to the results shown in Figs. 2 and 4, the D2R agonist quinpirole increased leptin and IL-6 protein expression (leptin: 1 μM, 24 h, +59 ± 4%; 10 μM, 24 h, +31 ± 8%; and IL-6: 1 μM, 24 h, +76 ± 25%; 10 μM, 24 h, +20 ± 24%) in the presence of insulin (supplementary Fig. S1). However, the basal level was...
Dopamine decreases leptin expression via adrenergic receptors in 3T3-L1 cells

It has been reported that dopamine suppresses leptin release in 3T3-L1 cells and human subcutaneous adipocyte tissue (10–12). However, our data show that D1R increases leptin expression (Figs. 2, 4, and supplementary Fig. S1). Therefore, to address the discrepancies between our results and those of others, we treated 3T3-L1 cells with dopamine in the presence of D1R antagonist, D2R antagonist, α-adrenergic receptor antagonist, or β-adrenergic receptor antagonist, with or without insulin (Fig. 5). We found that dopamine (1 μM, 24 h) decreased leptin expression (−33 ± 3% vs. control) but the D1R antagonist SCH 23390 (10 μM) did not prevent the inhibitory effect of dopamine on leptin expression. However, the presence of the D2R antagonist L741,626 enhanced the inhibitory effect of dopamine on leptin expression (−84 ± 4% vs. dopamine alone), suggesting that the D2R increases leptin expression. The β-adrenergic antagonist propranolol (10 μM) did not clearly affect the inhibitory action of dopamine on leptin expression. By contrast, the α-adrenergic antagonist phentolamine (10 μM) prevented the inhibitory effect of dopamine on leptin expression. This finding, however, is somewhat different from that reported by Than et al., who showed that the inhibitory effect of dopamine (1 μM) on leptin secretion was blocked by propranolol but not by phentolamine or the D1-like (SCH23390) or D2-like (haloperidol) receptor antagonists (11). We also found that dopamine decreased and the stimulatory effect of quinpirole on protein expression was no longer observed in the medium without insulin (leptin: vehicle −59 ± 16%, 1 μM 24 h, −60 ± 17%; 10 μM, 24 h, −58 ± 6% vs. control; and IL-6: vehicle, −23 ± 15%; 1 μM, 24 h, −46 ± 2%; 10 μM, 24 h, −57 ± 16% vs. control) (Fig. S1A). Leptin released in the medium was also decreased in the absence of insulin and D2R stimulation no longer had an effect on leptin released from 3T3-L1 cells (leptin in insulin-free media: vehicle, −37 ± 2%; 1 μM quinpirole, 24 h, −45 ± 2%; 10 μM quinpirole, 24 h, −33 ± 7%) vs. control (supplementary Fig. S1B). The stimulatory effect of quinpirole on leptin secreted into the medium was restored by the addition of leptin concentration used was at supraphysiological levels. Therefore, new experiments are needed to confirm the effect of insulin on the regulatory effects of D2R on leptin expression.

DISCUSSION

We and others have reported that the D2R has anti-inflammatory properties in the kidney (15, 16, 25) and
However, the function of the D2R in adipocytes is not clear. We now report that the D2R and D3R are expressed in mouse 3T3-L1 adipocytes, where the D2R upregulates the mRNA and protein expression of leptin and IL-6 via the PI3K/AKT pathway. The stimulatory effect of the D2R on leptin expression is dependent upon the presence of insulin.

It has been reported that D2-like receptors decreased the expression of adipokines such as leptin, IL-6, TNFα, and adiponectin (27). These observations were consistent with the concept of the existence of unique leptin-dopamine interactions in the hypothalamus and the hyposensitivity of the dopamine system in obesity, and may provide indirect evidence for an inhibitory effect of dopaminergic neurotransmission on leptin secretion via autonomic nerves. It has been described that the central part of the autonomic nervous system and intra-abdominal and subcutaneous fat pads are innervated by separate sympathetic and parasympathetic motor neurons (28). Moreover, some studies have shown that bromocriptine, a D2-like receptor agonist, decreases the leptin concentration in serum, likely via hypothalamic action (27, 29); therefore, systemic stimulation of D2R could decrease leptin expression. In contrast, Drd2−/− mice have lower serum leptin concentration compared with their wild-type littermates (30), indicating that additional mechanisms may be involved in the regulation of leptin by D2R. By contrast, female Drd3−/− mice have increased serum leptin concentration; a high-fat diet increases serum leptin concentration in both male and female Drd3−/− mice (31). Whether or not this dynamic regulation between dopamine receptors and leptin is organ-specific remains to be determined (32, 33).

Recently, other authors have proposed the hypothesis that the interaction between adipokines and dopamine also occurs in the adipocyte tissue. Brown, but not white, adipocytes (23) express dopamine receptors in some studies (34). One main question is, if dopamine receptors exert an effect on adipocyte regulation, what is the source of the dopamine in adipocyte tissue? It has been shown that human adipocytes possess arylsulfatase A, and circulating dopamine sulfate may serve as the source of dopamine in adipocytes (10).

Dopamine has been shown to decrease leptin expression in human subcutaneous adipocyte tissue (10, 12). 3T3-L1 cells may also express D2R (35) (Fig. 1). Dopamine and some dopamine receptor agonists are also able to stimulate α- and β-adrenergic receptors in the cardiovascular system and in adipocytes (11, 23, 24, 35, 36) and this effect in vivo is observed at a higher dopamine dose in humans (37). Dopamine also suppresses leptin release in 3T3-L1 cells but the effect is blocked, not by the dopamine receptor antagonists, but by propranolol, indicating the involvement of β-adrenergic receptors (11). White and brown adipose tissue of Sprague-Dawley rats can synthesize catecholamines (38).

Our data show that the D2R agonist increases leptin and IL-6 expression in 3T3-L1 cells. However, consistent with previous studies, our data show that dopamine decreases leptin expression mainly via β-adrenergic receptors in adipocytes.
3T3-L1 cells, while it increases leptin expression when α and β-adrenergic receptors are blocked by their antagonist (Fig. 5). Thus, the ability of dopamine to decrease leptin in subcutaneous adipocytes may be by the stimulation of adrenergic receptors, independent of dopamine receptors. Human D2R is expressed to a greater extent in visceral adipocytes than subcutaneous adipocyte tissue (Fig. 1), consistent with previous reports (10).

Dopamine and the D1-like receptor agonist SKF38393 decrease leptin release but increase adiponectin and IL-6 release in human adipocyte explants and differentiated primary adipocytes (10). Our data show that the D1R antagonist failed to block the dopamine effect on leptin expression (Fig. 5), suggesting that this action is independent of D1R, and that D2R may be involved in leptin regulation. SKF38393 can also stimulate adrenergic receptors (36), indicating that the capacity of SKF38393 to downregulate leptin expression may be independent of the dopamine receptors.

By contrast, Nakano et al. (13) showed that dopamine causes inflammation, inducing the expression of IL-6 and IL-17. The effect is blocked by the D1R antagonist in human primary cultures from synovial tissues, indicating that the possible pro-inflammatory effects of dopamine receptors may be an alternative target in inflammatory disease (39). In addition, a study of human subcutaneous adipocyte tissue has shown that dopamine receptors increase the expression of IL-6 (10).

Leptin increases pro-inflammatory cytokines such as IL-6 (2). Our data show that IL-6 is upregulated by D2R stimulation, which increases leptin release into the medium that is blocked by an OB-R receptor antagonist. Hence, leptin in the medium may increase the expression of pro-inflammatory adipokines via paracrine and autocrine action in 3T3-L1 cells via OB-R stimulation (40) (Fig. 7). Our in vivo data confirm that stimulation of D2R with quinpirole increases leptin synthesis from visceral adipocyte tissue because there is a remarkable increase in its mRNA expression. In addition, stimulation of D2R may also increase leptin release into the blood because the increase in serum leptin concentration occurred within 2 h of the treatment. Therefore, the stimulatory effect of D2R on leptin is confirmed in vitro and in vivo, which may have pro-inflammatory effects in adipocytes.

Taking all this into account, the physiological role of D2R in adipocytes in the regulation of leptin in vivo is complicated; hypothalamic D2R could be also involved in the leptin regulation (28). Because leptin could contribute to the inflammatory effect of visceral adipocyte tissue, we speculate that the stimulatory effect of D2R on leptin may have special relevance in the study of insulin-resistant diabetic patients or in patients under D2R agonist treatment. D2R-altering drugs are prescribed to patients with nervous and psychiatric disorders, hyperprolactinemia, or in the early phase of Parkinson’s disease. This is the first report that shows that D2R in adipocytes could have pro-inflammatory effects. Visceral adipocytes have also been reported to increase the expression of pro-inflammatory adipokines that could alter liver triglyceride metabolism and increase the development of arteriosclerosis (5). However, recent reports have shown that pro-inflammatory responses in adipocyte tissue are essential for proper extracellular matrix remodeling and angiogenesis (41). Therefore, whether this mechanism has significant physiological benefits or deleterious consequences must be determined. The understanding of the action of these drugs and undesirable side-effects, especially in viscerally obese patients, may be improved by considering their ability to directly affect adipocyte function.

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