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PAPER

Hormonal regulation of interleukin-6 production in human adipocytes

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OBJECTIVE: To elucidate the hormonal regulation of interleukin-6 (IL-6) production by human adipose tissue and its relation to leptin.
DESIGN: *In vitro* study. Human adipocytes were incubated with dexamethasone (with or without RU486), norepinephrine and epinephrine (with or without propranolol), or insulin.
MEASUREMENTS: IL-6 and leptin secretion by human adipocytes.
RESULTS: A gradual increase in IL-6 secretion by adipocytes during differentiation was observed. A positive correlation was found between basal IL-6 release and both glycerol 3-phosphate dehydrogenase activity — a marker of adipocyte differentiation — and leptin release. Dexamethasone decreased IL-6 secretion and increased leptin secretion in a dose-dependent manner. Both catecholamines increased IL-6 and leptin secretion. The effects of dexamethasone and catecholamines on IL-6 and leptin were abrogated by RU486 and propranolol, respectively. Incubation with insulin resulted in a dose-dependent stimulation of IL-6 and leptin secretion.
CONCLUSION: IL-6 is produced by human adipocytes and is a potential marker of adipocyte differentiation. Furthermore it is a hormonally regulated cytokine, suppressed by glucocorticoids, and stimulated by catecholamines and insulin in physiological concentrations.
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Keywords: interleukin-6; adipocyte; insulin; cortisol

Introduction

Interleukin-6 (IL-6) is a multifunctional cytokine with endocrine and metabolic actions, produced by many types of cells, including those of endocrine organs.[1] Accumulating evidence suggests that along with leptin, which belongs to the IL-6 family of cytokines, adipocytes produce and secrete significant amounts of IL-6 in the systemic circulation.[2] In fact, plasma IL-6 concentration correlates positively with body mass index (BMI) in humans,[3] suggesting that the adipose tissue is a major determinant of circulating IL-6 in states of obesity. Several epidemiological studies have identified IL-6 and C-reactive protein (CRP) as predictors of general morbidity in the elderly.[4] IL-6 and CRP are prognostic factors in unstable angina[5,6] and are associated with cardiovascular and all-cause mortality.[7] Visser *et al* showed in a large epidemiological study that overweight adults have constitutively elevated serum CRP concentration.[8] Based on the fact that CRP concentration is primarily determined by IL-6,[9,10] and in light of earlier studies showing that the adipose tissue is a major source of circulating IL-6, these investigators inferred that 'a state of low-grade systemic inflammation is present in overweight and obese persons'. Thus IL-6 derived by the adipose tissue may be playing a pivotal role in the pathogenesis of the cardiovascular disease typically associated with obesity.[11]

To further understand the physiology of IL-6 release by fat and its relation to leptin, we studied the hormonal regulation of IL-6 secretion by human adipocytes. Specifically we focused on the effects of the fat-acting hormones, insulin, glucocorticoids and catecholamines, on IL-6 and leptin

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secretion, as well as the correlation between indices of adipogenesis and IL-6 release.

Materials and methods

Differentiation of preadipocytes to adipocytes

Human preadipocytes (Zen-Biotechnology, Research Triangle Park, NC, USA) were plated in 24-well plates and maintained in Dulbecco's Modified Eagles Medium/F-10 supplemented with fetal bovine serum (10%) and Hepes (15 mM) in 5% carbon dioxide atmosphere (CO_2) at 37°C; the media was changed every 3 days. When cells became 90% confluent the differentiation to adipocytes was induced as follows. Cells were incubated in Dulbecco's Modified Eagles Medium/F-10 supplemented with fetal bovine serum (3%), biotin (33 μM), pantothenate (17 μM), insulin (100 nM), dexamethasone (1 μM), isobutylmethylxanthine (0.20 mM) and a PPAR-γ agonist in 5% carbon dioxide (CO_2) atmosphere at 37°C for 3 days. Afterwards the media was changed to 'adipocyte media': Dulbecco's Modified Eagles Medium/F-10 supplemented with fetal bovine serum (3%), biotin (33 μM), pantothenate (17 μM), insulin (100 nM) and dexamethasone (1 μM). The media was changed every 3 days for a total of 2 weeks. At that point supernatants were collected and stored at −80°C for measurement of IL-6 and leptin concentrations. To assess the time-course of IL-6 production during differentiation of preadipocytes to adipocytes, the supernatants of two wells were collected longitudinally every 3 days for a total of 34 days. IL-6 and leptin concentrations were determined by ELISA (R&D Systems, Minneapolis, MN, USA). The interassay coefficient of variation (CV) was 2 and 3.5% for IL-6 and leptin, respectively; the intraassay CV was 1.7 and 3.2% for IL-6 and leptin, respectively.

Adipocyte cultures

Human preplated adipocytes (Zen-Biotechnology, Research Triangle Park, NC, USA) were cultured in Dulbecco's Modified Eagles Medium/F-10 supplemented with fetal bovine serum (3%), biotin (33 μM), pantothenate (17 μM), insulin (100 nM) and dexamethasone (1 μM) in 5% CO_2 atmosphere at 37°C.

Hormone experiments

Hormones and hormone receptor antagonists were obtained from Sigma (Sigma, St Louis, MO, USA). Before each experiment cells were cultured in hormone-free F-10 medium supplemented with fetal bovine serum (3%), biotin (33 μM) and pantothenate (17 μM) for 48 h. Afterwards cells were incubated with insulin (10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M), dexamethasone (10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M), norepinephrine (10^{-10}, 10^{-8} and 10^{-7} M), or epinephrine (10^{-10}, 10^{-8} and 10^{-7} M) in separate experiments. The effects of catecholamines were studied in the presence or absence of the β-adrenergic receptor antagonist propranolol (10^{-6} M). Adipocytes were also incubated with the glucocorticoid receptor antagonist RU486 (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M) in the presence of dexamethasone (10^{-6} M). In all of the above experiments cells were incubated in 5% CO_2 atmosphere at 37°C for 48 h. At the end of each experiment supernatants were collected and stored at −80°C for measurement of IL-6 and leptin concentrations.

Assessment of glycerol 3-phosphate dehydrogenase (GPDH) activity

Adipocyte differentiation was assessed by measuring the activity of the marker of adipogenesis, GPDH.[12] Cells were homogenized using a lysis buffer (1 M Tris pH 7.5, 5 M NaCl, Triton 20%, 0.5 M EDTA, 100 mM Na_3 VO_4, leupeptin, and aprotinin). Cell suspensions were centrifuged at 14000 rpm for 20 min at 4°C and supernatants were removed and assayed for GPDH activity immediately. GPDH activity was measured as previously described.[13] Briefly, the reaction mixture (100 nM triethanolamine/HCl buffer, 2.5 mM EDTA, 0.12 mM NADH, 0.2 mM dihydroxyacetone phosphate, and 0.1 mM β-mercaptoethanol) was added to the supernatants. Enzymatic activity was assessed by spectrophotometry at 25°C. One unit of enzyme activity corresponded to the oxidation of 1 nmol of NADH/min. Results were corrected for protein concentration.

Protein concentration assay

Protein determination was performed by using the reagents provided by Pierce Chemical Co. (Rockford, IL, USA), according to the instructions provided by the manufacturer.

Statistical analysis

All experiments were performed in triplicates. The results are expressed as the mean ± s.e.m. Statistical analysis was performed by using the Mann–Whitney test; correlations were assessed by the Spearman Rank Order Test. A *P*-value less than 0.05 was considered statistically significant.

Results

Effects of hormones on IL-6 and leptin production by adipocytes

Dexamethasone. Incubation with dexamethasone resulted in an inhibition of IL-6 production by adipocytes in a dose-dependent fashion. The lowest effect was observed at 10^{-8} M (from 5600 ± 516 pg/10^6 cells to 3100 ± 200 pg/10^6 cells; $P = 0.0001$); the maximum effect of dexamethasone was observed at 10^{-6} M (2017 ± 133 pg/10^6 cells; $P < 0.0001$ *vs* baseline, Figure 1A).

On the contrary, treatment of adipocytes with dexamethasone led to a dose-dependent increase in leptin secretion. The lowest effect was detected at 10^{-12} M (from baseline of 7067 ± 500 pg/10^6 cells to 11516 ± 1050 pg/10^6

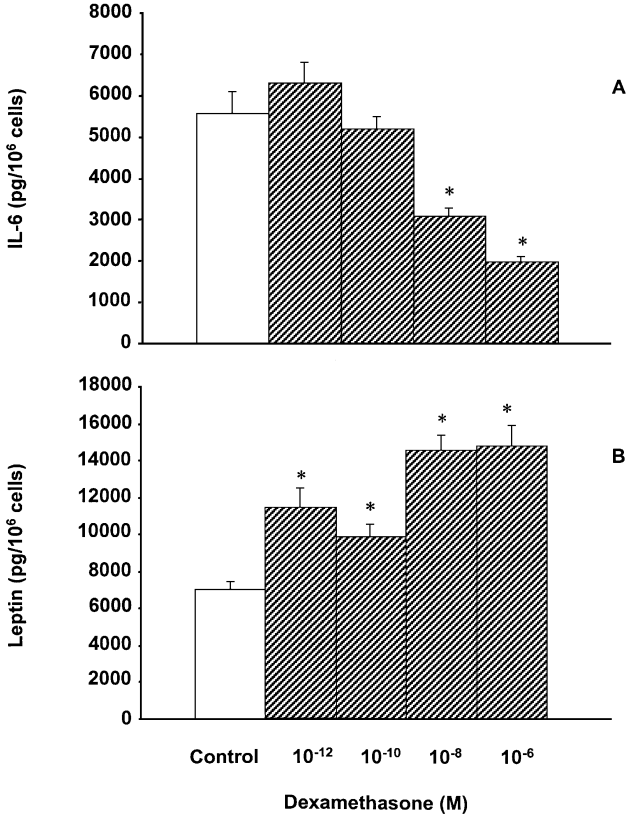

Figure 1 Effects of dexamethasone on interleukin-6 (IL-6) and leptin secretion by human adipocytes. Dexamethasone decreased IL-6 secretion (*A*) and increased leptin secretion (*B*) in a dose-dependent manner. *$P < 0.05$ or less *vs* control.

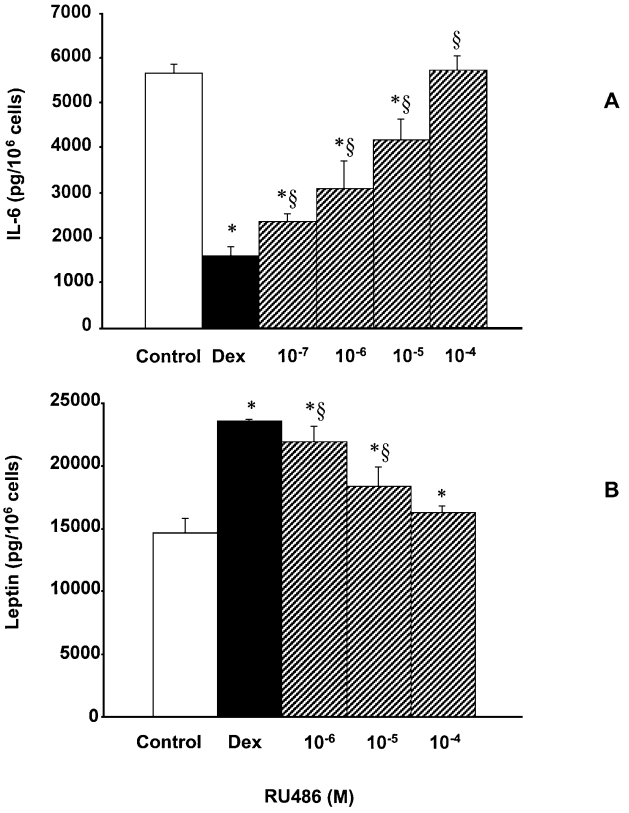

Figure 2 Effects of the glucocorticoid receptor antagonist RU486 on IL-6 (*A*) and leptin (*B*) secretion by adipocytes incubated with dexamethasone. *Black bars* indicate incubation with dexamethasone (10^{-6} M; Dex) in the absence of RU486. *Hatched bars* indicate incubation with dexamethasone (10^{-6} M) in the presence of incremental doses of RU486. *$P < 0.05$ or less *vs* control. §$P < 0.05$ or less *vs* Dex.

cells; $P < 0.05$). The highest effect was observed at 10^{-6} M (14817 ± 1150 pg/10^6 cells; $P < 0.0001$ *vs* baseline; Figure 1B). Incubation of adipocytes with the glucocorticoid antagonist RU486 inhibited the effects of dexamethasone (10^{-6} M) on both IL-6 and leptin production in a dose dependent manner. The effects of dexamethasone were abrogated with RU486 10^{-4} M. (Figure 2).

Catecholamines. Incubation with norepinephrine resulted in an increase of IL-6 production in a dose-dependent manner; the lowest effective dose was 10^{-10} M (from baseline of 3100 ± 233 pg/10^6 cells to 4333 ± 966 pg/10^6 cells; $P < 0.05$). The highest effect was observed at 10^{-8} M (9933 ± 2517 pg/10^6 cells; $P < 0.005$ *vs* baseline; Figure 3A). Similarly, leptin production was stimulated by norepinephrine 10^{-8} M, albeit to a lesser degree (from baseline of 3650 ± 683 pg/10^6 cells to 5450 ± 583 pg/10^6 cells; $P < 0.05$; Figure 3B).

Epinephrine induced a dose-dependent increase in IL-6 production. The lowest effective dose was 10^{-10} M (11267 ± 3316 pg/10^6 cells; $P < 0.005$ *vs* baseline); the maximum effect was observed at 10^{-8} M (12833 ± 4566 pg/10^6 cells; $P < 0.02$ *vs* baseline; Figure 4A). Similar to norepinephrine, epinephrine stimulated leptin production by adipocytes; the lowest effective dose was 10^{-10} M (5233 ± 466 pg/10^6 cells; $P < 0.03$ *vs* baseline). The highest effective dose was 10^{-7} M (6917 ± 300 pg/10^6 cells; $P < 0.005$ *vs* baseline; Figure 4B). Propranolol blunted the effects of both norepinephrine and epinephrine on IL-6 and leptin secretion by adipocytes (Figures 3 and 4).

Insulin. Incubation of mature adipocytes with insulin was followed by an increase of IL-6 secretion in a dose-dependent manner. The lowest effective insulin dose was 10^{-9} M (from a baseline of 4800 ± 700 pg/10^6 cells to 6183 ± 200 pg/10^6 cells; $P < 0.05$), whereas the greatest effect was observed at 10^{-6} M (7275 ± 716 pg/10^6 cells; $P < 0.05$ *vs* baseline; Figure 5A).

As expected, insulin stimulated leptin secretion in a dose-dependent manner. The lowest effective insulin dose was 10^{-9} M (from a baseline of 9867 ± 1100 pg/10^6 cells to 16083 ± 866 pg/10^6 cells; $P = 0.009$ *vs* baseline); the maximum effect was observed at 10^{-6} M (17967 ± 1233 pg/10^6 cells; $P = 0.003$ *vs* baseline; Figure 5B).

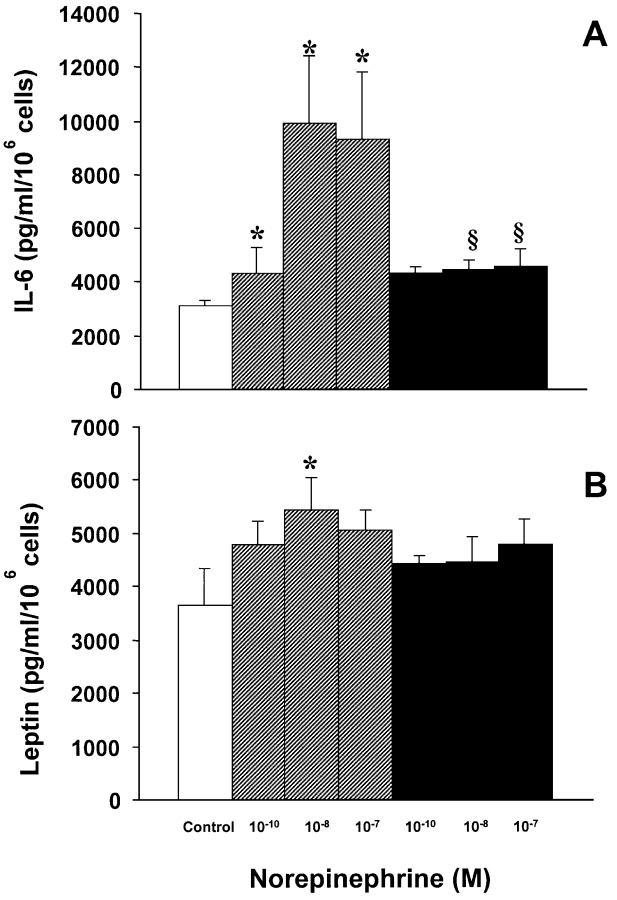

Figure 3 Effects of norepinephrine on IL-6 (*A*) and leptin (*B*) secretion by adipocytes in the presence (*black bars*) or absence (*hatched bars*) of propranolol (10^{-6} M). *$P < 0.05$ or less *vs* control. §$P < 0.05$ or less *vs* treatment without propranolol.

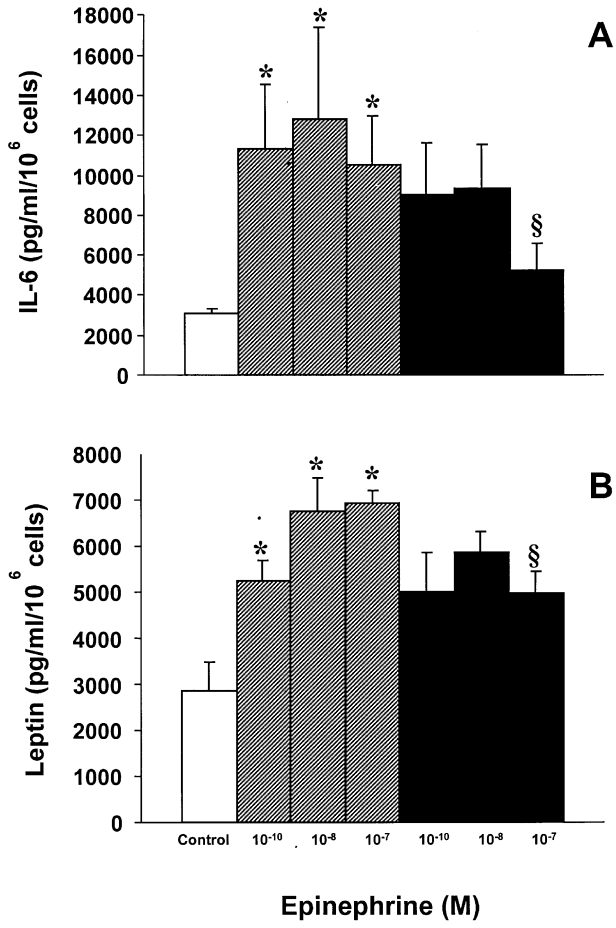

Figure 4 Effects of epinephrine on IL-6 (*A*) and leptin (*B*) secretion in the presence (*black bars*) or absence (*hatched bars*) of propranolol (10^{-6} M). *$P < 0.05$ or less *vs* control. §$P < 0.05$ or less *vs* treatment without propranolol.

IL-6 and leptin production during adipocyte differentiation

A positive correlation was found between GPDH activity and both IL-6 ($r = 0.44$; $P = 0.002$) and leptin ($r = 0.35$; $P = 0.03$). A positive correlation was found between IL-6 and leptin as well ($r = 0.57$; $P = 0.04$; Figure 6). A gradual increase of IL-6 secretion by adipocytes during differentiation was observed in the time-course experiment (Figure 7).

Discussion

This study is among the first to address the hormonal regulation of IL-6 release by human adipocytes. Dexamethasone suppressed release of IL-6 in a dose-dependent manner, in concordance with earlier studies.[14,15] Dexamethasone stimulated leptin secretion by adipocytes in agreement with earlier studies as well.[16,17] Both effects of dexamethasone were inhibited in the presence of RU486, suggesting that they were mediated via the glucocorticoid receptor.

Norepinephrine and epinephrine stimulated IL-6 and leptin secretion by adipocytes. Both of these effects were inhibited by the β-adrenergic receptor antagonist propranolol, suggesting that catecholamines exert their action on adipocytes mainly through β-adrenergic receptors. The stimulatory effects of catecholamines on IL-6 secretion through stimulation of the β-receptor are in concordance with earlier studies in animals and humans.[18,19] Our findings are also in agreement with the findings of Päth *et al*, who found that the specific β-adrenergic receptor agonist, isoproterenol, stimulated IL-6 secretion by human breast adipocytes *in vitro* in a dose-dependent manner.[14] However, unlike earlier studies that have suggested that catecholamines decrease leptin production,[20,21] we found that both epinephrine and norepinephrine stimulated leptin release in our system. The majority of earlier studies showing that catecholamines have an inhibitory effect on leptin have been conducted *in vivo*. Therefore they may reflect indirect actions of catecholamines on circulating leptin concentrations, through an increase in circulating free fatty acids, known to suppress leptin production,[22] rather than a direct action on

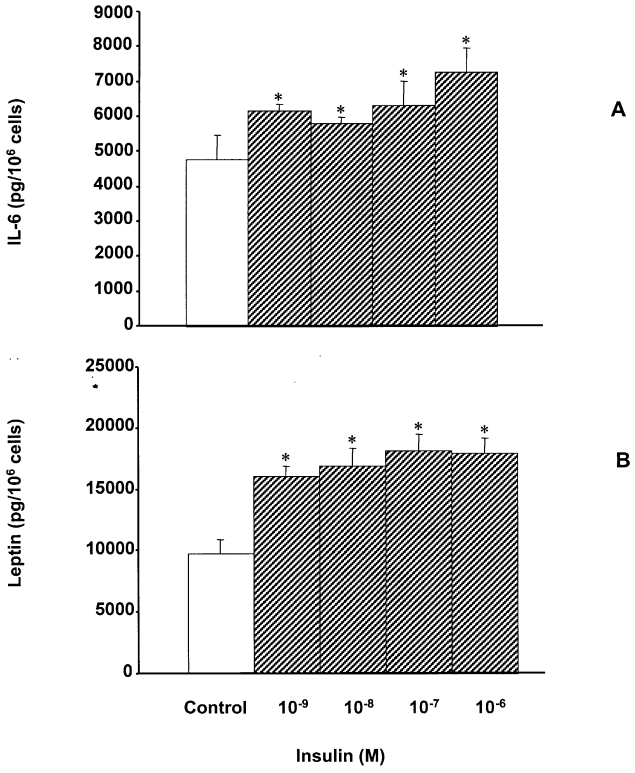

Figure 5 Effects of incremental doses of insulin on IL-6 (*A*) and leptin secretion (*B*). *$P < 0.05$ or less *vs* control.

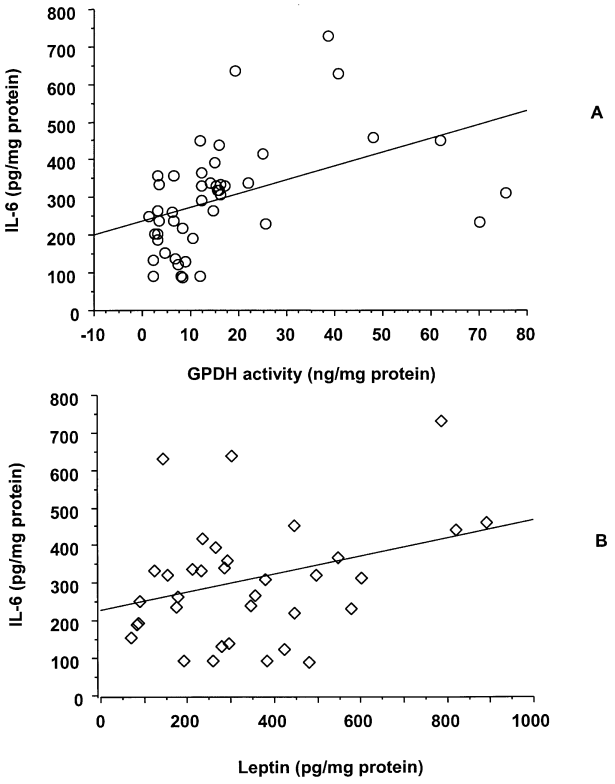

Figure 6 Correlation between basal IL-6 secretion and glycerol 3-phosphate dehydrogenase (GPDH) activity (*A*) and between basal IL-6 secretion and leptin secretion (*B*) in human adipocytes.

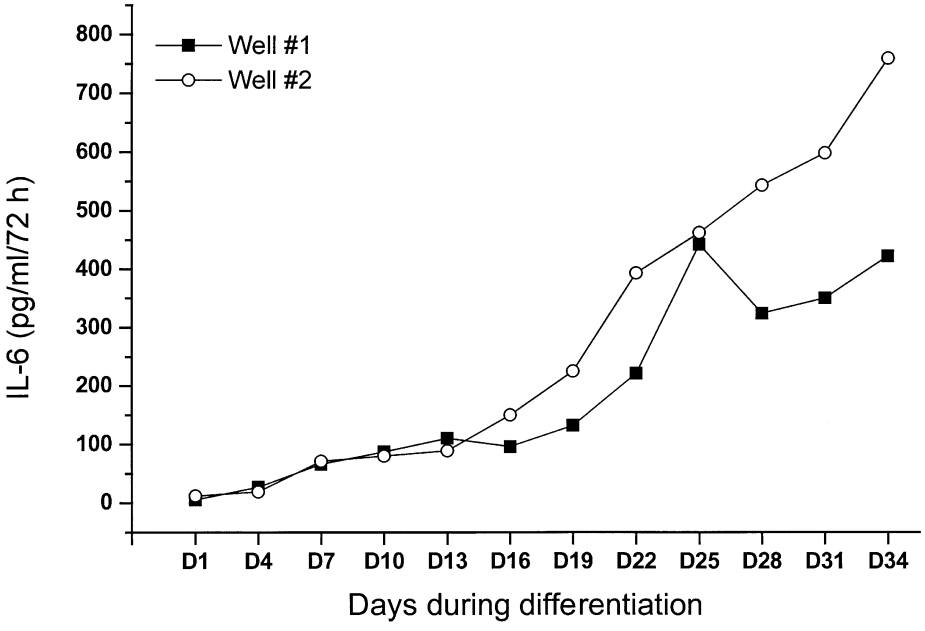

Figure 7 IL-6 production by adipocytes during differentiation. Supernatant was collected every 3 days. D1: first collection day (ie after 3 days of incubation of cells with 'adipocyte media'; see Materials and methods).

adipocytes. Several *in vitro* studies have also shown that catecholamines decrease leptin secretion by rodent adipocytes.[23,24] We found similar results when we applied higher doses of catecholamines (10^{-6} M). However, such doses were lethal to our cells (confirmed by the absence of any GPDH activity in cells treated with either catecholamine at 10^{-6} M); in fact not only did we observe a decrease in leptin, but also a paradoxical decrease in IL-6 concentration as well (data not shown). Therefore, the decrease in IL-6 and leptin release following high doses of catecholamines should be attributed to cell death, rather than to a decrease in leptin and cytokine production and/or release *per se*. The cytotoxic dose of catecholamines obviously varies among species.

The present study extends our earlier observations that IL-6 concentrations correlate positively with BMI.[3,25] Specifically, we found that basal IL-6 secretion by adipocytes was gradually increased during adipogenesis and that it correlated positively with two different markers of adipogenesis, namely leptin and GPDH. Furthermore, we found that insulin stimulated IL-6 production in adipose cells in a dose-dependent manner. This is the first study to demonstrate that insulin stimulates IL-6 production by adipocytes, and can potentially provide an explanation for the elevated plasma IL-6 concentration observed in obesity, which is often characterized by hyperinsulinemia. Insulin has been implicated as a major factor in the pathogenesis of coronary artery disease.[26] Our findings support the notion that these effects of insulin may be mediated via an increase in IL-6 production by fat. This might explain the correlation between plasma IL-6 concentration and features of the insulin resistance syndrome reported in healthy subjects.[27]

Is there a physiologic role for the stimulation of IL-6 by insulin? Like leptin, IL-6 is anorexiogenic.[28,29] Thus it appears that, in physiological conditions, insulin would stimulate adipocytes to produce leptin (the satiety hormone) and IL-6 (the anorexiogenic factor), both of which would, in turn, act centrally to reduce food intake and increase body expenditure.[29–31]

In conclusion we have shown that IL-6 is produced by mature human adipocytes and is in fact a potential marker of adipocyte differentiation. Furthermore we have shown that it is a hormonally regulated endocrine cytokine, greatly stimulated by insulin in physiological concentrations. The potential pathophysiologic implications of these findings in states of obesity and/or hyperinsulinemia merit further study.

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