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Insulin enhances vascular endothelial growth factor, interleukin-8, and plasminogen activator inhibitor 1 but not interleukin-6 release by human adipocytes

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Abstract

The present studies were designed to investigate the hormonal regulation of vascular endothelial growth factor (VEGF) release by human subcutaneous adipose tissue explants and adipocytes incubated in primary culture for 48 hours. Vascular endothelial growth factor and IL-8 release by adipocytes were less than 10% of that by tissue explants, whereas that of leptin in adipocytes was comparable to that by tissue. Dexamethasone inhibited VEGF formation by both adipose tissue explants and isolated adipocytes, whereas insulin stimulated VEGF release only in isolated adipocytes. Insulin also enhanced the formation of IL-8 and plasminogen activation inhibitor 1 (PAI-1), but not that of IL-6 by adipocytes although having little effect on that of IL-6 or PAI-1 by adipose tissue explants. Pertussis toxin stimulated lipolysis and inhibited leptin release by human adipose tissue or adipocytes but did not affect release of IL-8 or VEGF. Isoproterenol also stimulated lipolysis by human adipocytes, but this was not accompanied by any significant changes in VEGF, IL-8, IL-6, or PAI-1 release. In contrast, insulin stimulated VEGF release by human adipocytes, and this stimulation was enhanced in the presence of isoproterenol. Insulin stimulated VEGF formation as well as that of PAI-1 by human adipocytes, but not by explants under conditions where it had little effect on that of IL-6. The ability of insulin to stimulate VEGF formation by adipocytes suggests that the elevated circulating levels of insulin in obesity promote angiogenesis in adipose tissue as well as the enhanced accumulation of fat in human adipocytes.

1. Introduction

Vascular endothelial growth factor-A (VEGF) is a paracrine factor that is involved in angiogenesis [1,2]. Rupnick et al [3] reported that the angiogenesis inhibitor TNP-470 reduced the development of obesity in *ob*/*ob* mice and induced adipose endothelial cell apoptosis. Fukumura et al [4] similarly found that murine preadipocytes implanted in dorsal skin chambers developed into adipose tissue and a VEGF receptor-2 blocking antibody blocked this process. It is known that VEGF is a hypoxia-induced factor released by endothelial and/or smooth muscle cells. Claffey et al [5] reported that murine preadipocyte cell lines express and secrete VEGF during differentiation into adipocytes. Furthermore, agents that elevate cyclic adenosine monophosphate (AMP) or activate protein kinase C enhanced the expression of VEGF mRNA [5]. Vascular endothelial growth factor is released by human adipocytes although the amount released over 48 hours in primary culture was less than 6% of that by the nonfat cells from the same amount of tissue [6]. This may be an underestimation of adipocyte contribution because release of VEGF by human adipocytes was 27% that by human adipose tissue explants during the first 4 hours, as compared to 13% over 24 hours and 9% over 48 hours of incubation [6].

Zhang et al [7] reported that adipocytes rather than the stromal-vascular (SV) cells were the source of the VEGF released by rat visceral (omental) adipose tissue. In contrast, Mick et al [8] found that both the nonfat cells in rat adipose tissue and adipocytes produce VEGF, but only VEGF formation by adipocytes was stimulated by insulin. Vascular endothelial growth factor formation by rat adipocytes was unaffected by dexamethasone and stimulated by lipolytic agents such as norepinephrine, forskolin, and dibutyryl cyclic AMP [8]. The present studies were designed to see if hormones affect VEGF formation by human adipocytes and to compare the hormonal regulation

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of VEGF with that of plasminogen activation inhibitor 1 (PAI-1), IL-6, and IL-8.

2. Materials and methods

Abdominal subcutaneous adipose tissue was obtained from women who were undergoing laparoscopic gastric bypass with Roux-en-Y gastroenterostomy surgery for the treatment of morbid obesity. The study had the approval of the local institutional review board and all patients involved gave their informed consent. The patients were on a clear liquid diet the day before surgery, but had not been on any type of dietary restriction before surgery.

Samples of abdominal subcutaneous adipose tissue were immediately transported to the laboratory. The handling of tissue and cells was done under aseptic conditions. The tissue was cut with scissors into small pieces (10-30 mg). All the studies used explants of adipose tissue that had been incubated in buffer plus albumin (3 mL/g of tissue) for approximately 30 minutes to reduce contamination of the tissue with blood cells and soluble factors. At the conclusion of the 30-minute incubation, the tissue explants were centrifuged for 30 seconds at 400 × *g* to remove blood cells and pieces of tissues containing insufficient adipocytes to float. The explants (0.1 g/mL) were incubated in duplicate for the indicated times in suspension culture under aseptic conditions.

Adipocytes were prepared by incubating cut pieces of adipose tissue (0.5 g/mL in incubation medium containing 0.6 mg of bacterial collagenase per milliliter) in a rotary water bath shaker (100 rpm) for 2 hours [9,10]. The collagenase digest was separated from undigested tissue matrix by filtration through 200-μm mesh fabric. The adipocytes were suspended in buffer and centrifuged for 10 seconds at 400 × *g* then incubated at a concentration of 0.1 g/mL for the indicated periods.

The serum-free buffer for incubation of adipose tissue and adipocytes was as previously described [9,10]. The pH of the buffer was adjusted to 7.4 and then filtered through a 0.2-μm filter. Aliquots of the medium were taken at 48 hours and stored at −20°C for measurement of release to the medium. Leptin, VEGF-A_{165}, IL-6, and IL-8 were determined by ELISA assays using Duoset reagents from R&D Systems of Minneapolis in Minnesota. Plasminogen activation inhibitor 1 was measured using ELISA kits from American Diagnostica of Greenwich in Connecticut. Glycerol was measured using glycerol dehydrogenase [10]. All values are shown as the mean or the mean ± SEM.

Bovine serum albumin powder (Bovuminar, containing <0.05 mol of fatty acid per mole of albumin) was obtained from Intergen (Purchase, NY). Bacterial collagenase *Clostridium histolyticum* (type 1) was obtained from Worthington Biochemical Corporation (lot CLS1-4197-MOB3773-B, 219 U/mg). Other chemicals were from Sigma (St Louis, MO).

3. Results

Insulin (10 nM) significantly enhanced (by approximately 50%) the formation of VEGF by human adipocytes over 48 hours of incubation although having no effect on that by explants of human subcutaneous adipose tissue (Fig. 1). The release of PAI-1 was doubled by insulin in adipocytes

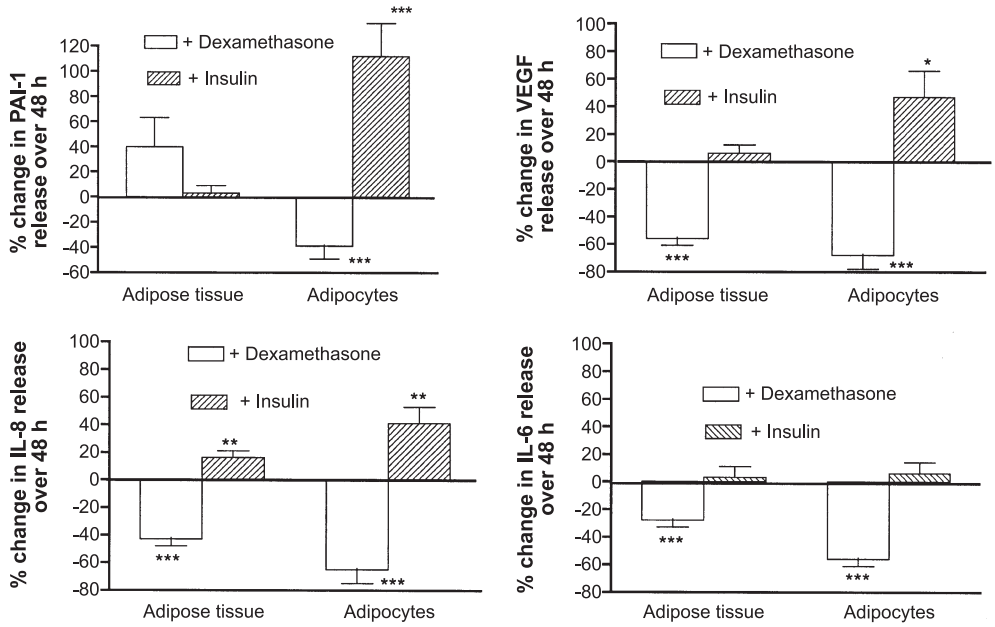

Fig. 1. Comparison of dexamethasone and insulin effects on VEGF and PAI-1 release by human adipose tissue explants and adipocytes. The data are shown as the mean ± SEM of the percent change in medium content due to insulin (10 nmol/L) or dexamethasone over that in the absence of the hormones for 6 to 17 paired experiments (6 for IL-6 release, 7 for PAI-1 release, 13 for VEGF release, and 17 for IL-8 release using subcutaneous adipose tissue or adipocytes incubated for 48 hours). Statistically significant effects or insulin or dexamethasone are indicated as follows on the basis of paired comparisons: *$P < .05$; **$P < .005$, and ***$P < .001$.

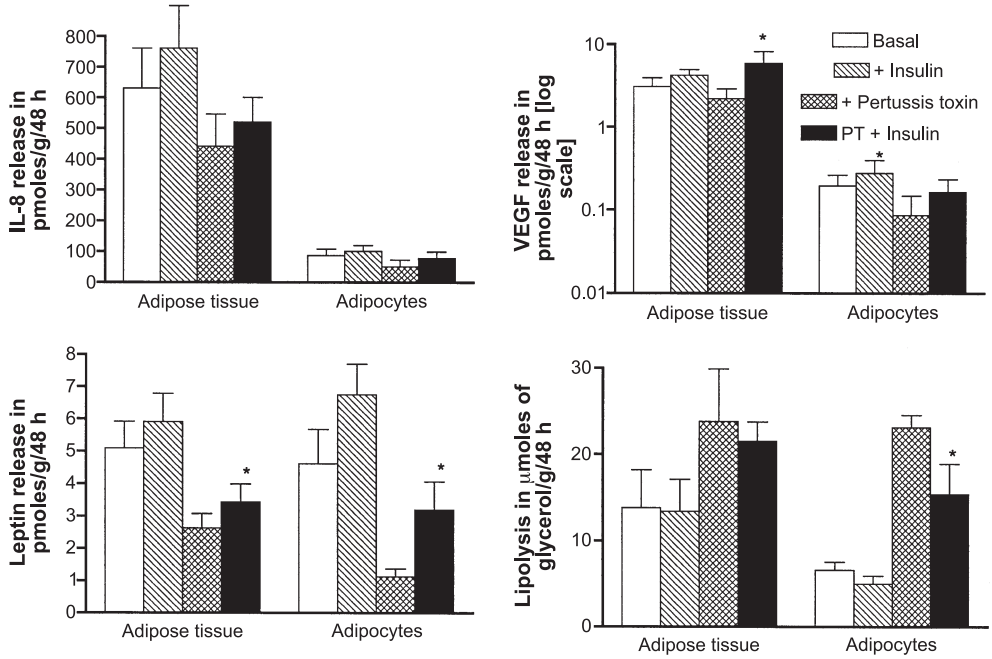

Fig. 2. Effect of pertussis toxin and insulin on VEGF, IL-8, and leptin release as well as lipolysis by human adipose tissue explants and adipocytes. The data are shown as the mean ± SEM of 7 paired experiments using subcutaneous adipose tissue or adipocytes incubated for 48 hours either with or without pertussis toxin (100 ng/mL) or with insulin (10 nmol/L). Statistically significant effects of insulin are indicated as follows on the basis of paired comparisons: $*P < .05$.

although, just as for VEGF, no stimulation was seen in adipose tissue explants. Insulin did not affect IL-6 release by either adipocytes or explants but did stimulate IL-8 release by both adipocytes and explants. However, the effect of insulin on IL-8 release by adipose tissue explants was only a 16% increase over basal. In contrast, dexamethasone significantly inhibited the release of PAI-1, VEGF, IL-6, and IL-8 by adipocytes and of VEGF, IL-6, and IL-8 by

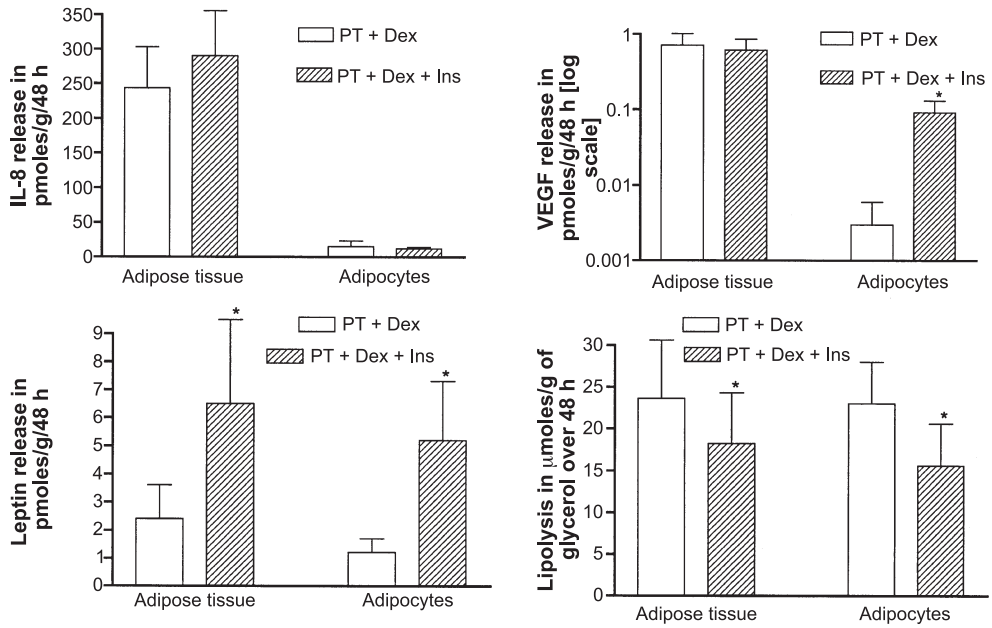

Fig. 3. Effect of pertussis toxin in the presence of dexamethasone on VEGF, IL-8, and leptin release as well as lipolysis by human adipose tissue explants and adipocytes in the absence or presence of insulin. The data are shown as the mean ± SEM of 7 paired experiments using subcutaneous adipose tissue or adipocytes incubated for 48 hours in the presence of dexamethasone (10 nmol/L) plus pertussis toxin (100 ng/mL) either in the absence or presence of insulin (10 nmol/L). Statistically significant effects of insulin are indicated as follows on the basis of paired comparisons: $*P < .05$.

explants. Dexamethasone is a synthetic glucocorticoid agonist, which was used because the release of many adipokines is affected by it in adipocytes or adipose tissue.

Mick et al [8] reported that lipolytic agents such as catecholamines, forskolin, or cell-permeable analog of cyclic AMP enhance VEGF release by rat adipocytes over a 16-hour incubation. Pertussis toxin, which adenosine diphosphate ribosylates and thus inactivates inhibitory guanine nucleotide binding protein (G_i), stimulates lipolysis [9]. Pertussis toxin stimulated lipolysis and inhibited leptin release in both adipocytes and tissue explants (Fig. 2) in confirmation of prior studies from our laboratory [9]. Furthermore, insulin stimulated leptin release in the presence of pertussis toxin by adipocytes while inhibiting lipolysis.

Neither IL-8 nor VEGF formation by adipose tissue or adipocytes was significantly affected by pertussis toxin (Fig. 2). Insulin stimulated VEGF release by adipocytes in the absence of pertussis toxin, but in the presence of toxin, the effect was more variable and not statistically significant. In explants of adipose tissue, just as in Fig. 1, there was no statistically significant effect of insulin on VEGF release, but a significant effect of insulin was seen in the presence of pertussis toxin. It should be noted that IL-8 and VEGF release by adipocytes over 48 hours was less than 10% of that by tissue as previously reported [6], whereas leptin release was comparable and lipolysis by adipocytes about half that by tissue.

The greatest percent increase in VEGF release due to insulin was seen in isolated adipocytes incubated in the presence of both pertussis toxin and dexamethasone, where VEGF release in the absence of insulin was very small (Fig. 3). However, no effect of insulin was seen in tissue explants where the formation of VEGF was 200-fold greater than in adipocytes. IL-8 release, in contrast, was not affected

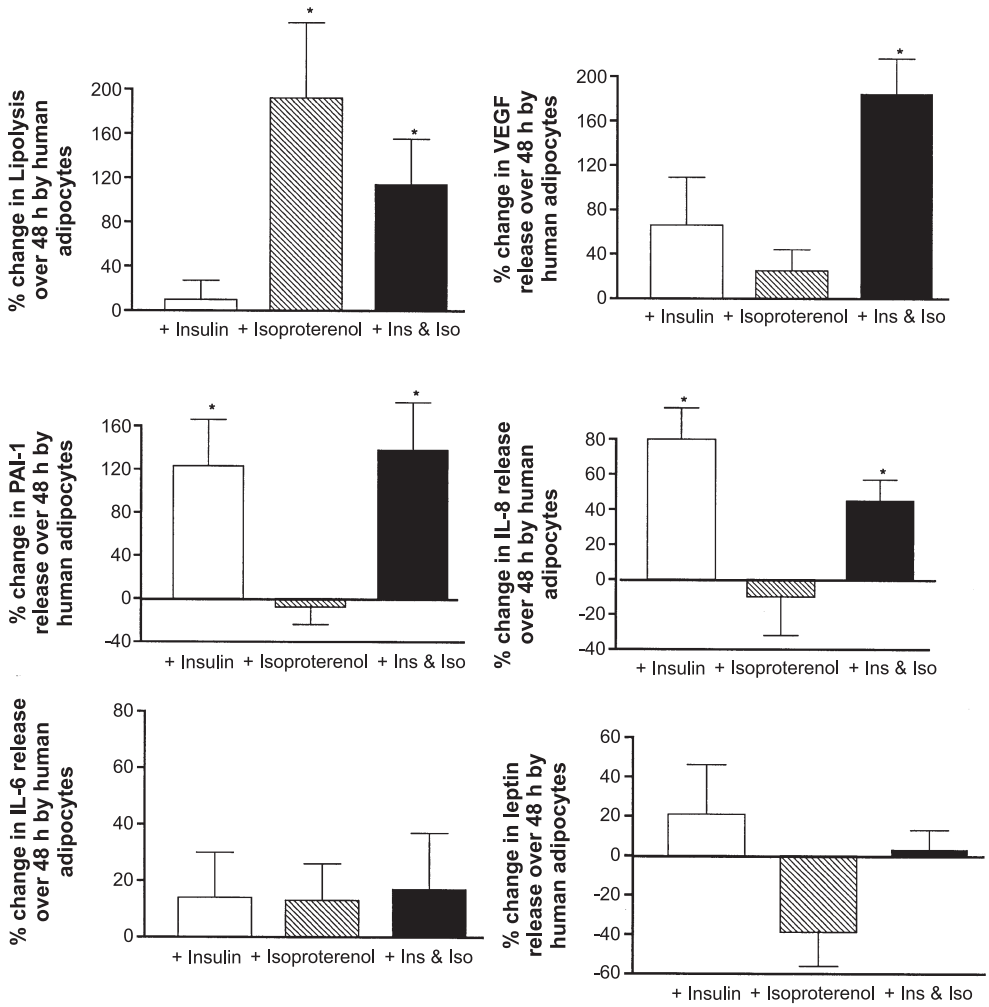

Fig. 4. Effect of isoproterenol and insulin on VEGF, IL-8, IL-6, PAI-1, and leptin release as well as lipolysis by human subcutaneous adipocytes. The data are shown as the mean ± SEM of the percent change from basal in 5 paired experiments using subcutaneous adipocytes incubated in the presence of dexamethasone (10 nmol/L) for 48 hours either with or without isoproterenol (10 nmol/L), with insulin (10 nmol/L) or both insulin and isoproterenol. The basal release by adipocytes incubated with dexamethasone was as follows: lipolysis, 3.1 μmol/g per 48 hours; VEGF, 0.046 pmol/g per 48 hours; PAI-1, 28 pmol/g per 48 hours; IL-8, 15 pmol/g per 48 hours; IL-6, 12 pmol/g per 48 hours; and leptin, 9 pmol/g. Statistically significant effects of added agents are indicated as follows on the basis of paired comparisons: *$P < .05$.

by insulin in adipocytes incubated in the presence of pertussis toxin plus dexamethasone, which were conditions where insulin stimulated VEGF and leptin release while inhibiting lipolysis.

Isoproterenol is a catecholamine that interacts with β receptors and stimulates lipolysis. Isoproterenol did not affect IL-6, IL-8, PAI-1, or VEGF release by human adipocytes incubated in the presence of dexamethasone (Fig. 4). Lipolysis was enhanced by isoproterenol and leptin was inhibited over the 48-hour incubation of adipocytes. However, in the presence of dexamethasone, the combination of insulin and isoproterenol significantly stimulated VEGF release although there was no significant effect of either agent alone. This was different from what was seen with PAI-1 where insulin enhanced its release to the same extent in the absence as in the presence of isoproterenol. In contrast, insulin stimulated IL-8 release and this effect was less in the presence of isoproterenol. IL-6 release, on the other hand, was unaffected by insulin, isoproterenol, or the combination.

4. Discussion

The present studies measured only VEGF-A_{165} release by human adipose tissue and adipocytes. There are several other members of the VEGF family (B, C, and D) but their function is unknown. Vascular endothelial growth factor B knockout mice are healthy and fertile but have cardiovascular changes [11]. The gene for VEGF-B is up-regulated 3.5-fold in visceral adipose tissue from obese [body mass index (BMI) = 37] as compared to lean (BMI = 23) human beings [12].

Vascular endothelial growth factor is secreted by both adipocytes and the SV or nonfat cells obtained by collagenase digestion of human adipose tissue [6]. Rehman et al [13] found that appreciable amounts of VEGF were released during primary culture by the nonfat cells (SV cells) isolated from human subcutaneous adipose tissue and that VEGF release was markedly enhanced by hypoxic conditions. Hypoxia apparently enhances VEGF release secondarily to the activation of a heterodimeric helix-loop-helix transcription factor that binds to a response element in the proximal VEGF promoter [14]. There is also a separate mechanism for activation of VEGF expression involving liver X receptor/retinoid X receptor heterodimers that has been seen in macrophages [14]. It is unclear exactly what the role of VEGF is in angiogenesis and whether the enhanced levels of insulin that are seen in obesity increase VEGF release by adipocytes and thus contribute to angiogenesis. However, our data indicate that adipocyte secretion of VEGF in response to insulin could play a role in the expansion of adipose tissue support vessels that clearly occurs in obesity.

We have reported that VEGF formation during a 48-hour incubation of human visceral adipose tissue explants was 3.5-fold greater than that by explants of subcutaneous adipose tissue from the same donors [6]. There was no significant difference among the release of VEGF by adipocytes isolated from visceral adipose tissue as compared to that by subcutaneous adipocytes [6]. This suggests that either collagenase digestion equalizes the difference between the 2 types of tissue or the difference is due to release of VEGF by nonfat cells present in adipose tissue. The failure of insulin to stimulate VEGF release by adipose tissue explants is probably due to the fact that formation by isolated adipocytes of VEGF was less than 10% that by intact tissue. A doubling of VEGF formation by adipocytes would be difficult to detect over the large background formation by nonfat cells in tissue explants.

The present results are in agreement with those of Mick et al [8] who found a stimulation of VEGF release by insulin in rat adipocytes but not that in isolated nonfat (stromal) cells over a 12-hour incubation. However, although dexamethasone did not inhibit VEGF formation by rat adipocytes [8], it clearly inhibited that by both human adipocytes and adipose tissue explants. A further difference was that lipolytic agents stimulated VEGF release by rat adipocytes although we were unable to find any stimulation of VEGF release by lipolytic agents in human adipocytes. However, we did find that a lipolytic agent (isoproterenol) stimulated VEGF release by human adipocytes if insulin was also present. In explants of adipose tissue, a small stimulation of VEGF release by insulin was seen only in the presence of pertussis toxin. Pertussis toxin stimulates lipolysis by a mechanism involving adenosine diphosphate ribosylation of a cysteine residue on the alpha subunit of the G_i protein that inhibits adenylate cyclase activation as first shown in human adipocytes by Kather et al [15]. In contrast, isoproterenol is a synthetic catecholamine that stimulates the β catecholamine receptors in adipose tissue and activates the guanine nucleotide binding protein, which activates adenylate cyclase.

The failure of dexamethasone to inhibit PAI-1 release by adipose tissue explants is comparable to the results published by He et al [16] who actually reported a transient stimulation at 8 hours by dexamethasone of PAI-1 release by explants of human subcutaneous adipose tissue. Morange et al [17] also found that dexamethasone stimulated PAI-1 release by human subcutaneous adipose tissue explants over a 16-hour incubation as did Halleux et al [18] over a 24-hour incubation. However, dexamethasone inhibited the increase in PAI-1 release by IL-1β [16]. Thus, the effects of dexamethasone may depend upon the absence or presence of cytokines such as IL-1β. Our finding that insulin did not stimulate PAI-1 release by explants was comparable to that by Morange et al [17]. In contrast, Halleux et al [18] reported a stimulation of PAI-1 release by explants due to insulin, and it may be that adipocytes accounted for most of the PAI-1 release by explants of adipose tissue incubated under their experimental conditions.

Juhan-Vague et al [19] suggested that the elevated levels of circulating insulin seen in obese subjects and especially those with type 2 diabetes might be responsible for the

elevated plasma levels of PAI-1 that are linked to increased coronary disease in these individuals. We were able to see a stimulation of human adipocyte PAI-1 release by insulin, and circulating levels of insulin are elevated in type 2 diabetes. However, the effect of insulin on PAI-1 formation are restricted to adipocytes in adipose tissue, but over 48 hours, the formation of PAI-1 by adipocytes was 25% of that by the nonfat cells from the same amount of adipose tissue [6]. In contrast, the formation by adipocytes of IL-8 was 12%, VEGF was 8%, and IL-6 was 7% of that by nonfat cells in adipose tissue [6]. Plasminogen activation inhibitor 1 circulates at concentrations (875 pmol/L in plasma) much higher than those of IL-6, IL-8, and VEGF whose plasma levels are less than 1 pmol/L [6]. Whether insulin stimulation of adipocyte PAI-1 release secondary to elevated levels of insulin in obesity and type 2 diabetes contributes to circulating levels of PAI-1 remains to be established.

Yudkin et al [20] have claimed that subcutaneous adipose tissue does not contribute significantly to circulating levels of PAI-1, but the role of visceral adipose tissue remains to be established. Furthermore, the release of PAI-1 by visceral adipose tissue has been found to be greater by human visceral than by subcutaneous adipose tissue explants [6,21]. Similar results have been reported for PAI-1 mRNA, and this was attributed to a greater content of stromal cells in visceral adipose tissue [22].

Halleux et al [18] reported that dibutyryl cyclic AMP (1 mmol/L), which is a lipolytic agent that activates protein kinase A, inhibited PAI-1 release by human visceral adipose tissue explants, adipocytes, or SV cells over a 4-hour incubation. Gottschling-Zeller et al [23] similarly reported that isoproterenol (1000 nmol/L) inhibited PAI-1 release over a 24-hour incubation. However, they found no significant inhibition by isoproterenol (100 nmol/L) and this is comparable to our finding with isoproterenol (10 nmol/L). High concentrations of lipolytic agents and added cyclic AMP probably increase AMP in human adipocytes as demonstrated by Fain [24] in rat adipocytes using norepinephrine in the presence of theophylline. An elevation in AMP leads to an activation of AMP kinase that switches on adenosine triphosphate–consuming processes and promotes catabolism [25]. Thus, the effects of lipolytic agents can be complex with activation of lipolysis occurring at low concentrations without any appreciable elevation of AMP, whereas at high concentrations, AMP can be elevated that in turn activates AMP kinase [25].

Krogh-Madsen et al [26] reported that the infusion of sufficient insulin to elevate plasma insulin in human beings to 0.8 nmol/L under euglycemic clamp conditions resulted in a transient increased in adipose tissue IL-6 mRNA content at 2 hours and a 3-fold increase in plasma IL-6 after 4 hours. It is unclear why we saw no effect of insulin on IL-6 release by human adipocytes incubated in vitro under conditions where it stimulated the release of IL-8, VEGF, and PAI-1. One explanation is that in vivo insulin infusion results in activation of IL-6 release through secondary effects.

The hormonal regulation of IL-8 release by explants of subcutaneous adipose tissue was examined by Bruun et al [27] who found that dexamethasone inhibited whereas IL-1β and tumor necrosis factor α stimulated IL-8 release over a 48-hour incubation. These authors reported no effect of insulin on IL-8 release although we found a small increase in tissue explants. However, appreciable increases in IL-8 release due to insulin were best seen in adipocytes incubated in the presence of dexamethasone. The regulation of IL-8 release apparently occurs by mechanisms distinct from those involved in the regulation of VEGF release, because in the presence of pertussis toxin plus dexamethasone, there was no effect of insulin of IL-8 release, but there was of VEGF release. Furthermore, isoproterenol that inhibited the stimulation of IL-8 release by insulin had no effect on that of PAI-1 while enhancing that of VEGF in human adipocytes. Our data indicate that the effects of insulin on the release of IL-8 and VEGF can be altered by the presence or absence of other hormones and may explain some of the variability in reported effects of insulin on adipokine release. Clearly, it is difficult to determine what the effects of insulin are on IL-8, PAI-1, and VEGF release by human adipose tissue in vivo because it is unclear what other hormones or regulatory factors are also present.

In conclusion, our data indicate that in isolated human adipocytes from individuals with extreme obesity incubated for 48 hours in primary culture, there is a stimulation by insulin of VEGF, PAI-1, and IL-8 release. Whether these stimulatory effects are present in vivo and physiologically important remains to be demonstrated. However, elevated levels of circulating insulin are seen in obese human beings. An enhanced secretion of VEGF, PAI-1, and IL-8 by adipocytes could conceivably contribute to the deleterious effects of obesity on cardiovascular disease.

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