Leptin Induces Angiopoietin-2 Expression in Adipose Tissues*

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Adipose tissues consisting of adipocytes, microvasculature, and stroma are completely ablated upon over-expression of leptin in rats. This tissue regression is mediated by enhanced lipid beta-oxidation, adipocyte dedifferentiation, and apoptosis. To further characterize this phenomenon, we studied the possible effect of leptin on the adipose microvasculature. Tissue microvasculature is maintained by the interplay between positive and negative signals mediated by factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor, angiopoietin-1 (Ang-1), and Ang-2. Expression of the negative signal Ang-2 was reported in fetal tissues and in the adult ovary, which undergoes vascular remodeling or regression. We demonstrate that leptin induces the expression of Ang-2 in adipose tissue without a concomitant increase in VEGF. Induction of Ang-2 occurred in an autocrine manner, as demonstrated in cultured adipocytes but not in several other cell types. This tissue-specific induction of Ang-2 coincided with initiation of apoptosis in adipose endothelial cells. We propose that induction of Ang-2 by leptin in adipose cells is one of the events leading to adipose tissue regression.

Leptin, a product of the obese (ob) gene, is a cytokine secreted by adipocytes, which regulates adipose tissue mass by restricting food intake and elevating the expenditure of metabolic energy (1, 2). These activities of leptin are mediated by enhanced lipid beta-oxidation, adipocyte dedifferentiation, and apoptosis. To further characterize this phenomenon, we studied the possible effect of leptin on the adipose microvasculature. Tissue microvasculature is maintained by the interplay between positive and negative signals mediated by factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor, angiopoietin-1 (Ang-1), and Ang-2. Expression of the negative signal Ang-2 was reported in fetal tissues and in the adult ovary, which undergoes vascular remodeling or regression. We demonstrate that leptin induces the expression of Ang-2 in adipose tissue without a concomitant increase in VEGF. Induction of Ang-2 occurred in an autocrine manner, as demonstrated in cultured adipocytes but not in several other cell types. This tissue-specific induction of Ang-2 coincided with initiation of apoptosis in adipose endothelial cells. We propose that induction of Ang-2 by leptin in adipose cells is one of the events leading to adipose tissue regression.

Leptin Induces Angiopoietin-2 and Apoptosis in Adipose Tissues—Swiss 3T3-F442A murine pre-adipo-cytes (24) were grown in DMEM with 10% calf serum. To induce differentiation into mature adipocytes, confluent cell cultures were maintained in DMEM supplemented with 10% fetal bovine serum for 6 days. Recombinant mouse leptin was purchased from R & D Systems (Minneapolis). Polyclonal antibodies to human Ang-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). An in situ cell death detection kit, AP, was purchased from Roche Molecular Biochemicals.

Immunohistochemistry—Subcutaneous fat was removed from various strains of mice at the indicated times and fixed with 4% paraformaldehyde, and paraffin sections were prepared. Apoptosis was determined in situ by a TUNEL assay on the paraffin sections using alkaline phosphatase and nuclei counted in five fields at ×200 (25). RNA Blot Analysis—Cells were maintained at low (0.5%) serum for 16 h and then treated with murine leptin (1 μg/ml) for different time periods. Total RNA was isolated either from adipose tissue or from cultured cells with the TRI reagent kit (Molecular Research Center Inc.). Aliquots (1.5 μg) were subjected to RNA blot analysis as described (26). Probes for RNA blot analysis were prepared by RT-PCR with total RNA and the following primers (GenBank™ accession numbers are in parentheses): muAng-2 mRNA (AF4326), nucleotides 637–657 and 1147–1167; muVEGF (M95200), nucleotides 385–406 and 962–980; muActin mRNA (J00691), nucleotides 2452–2431 and 1640–1620; huVEGF (M32977), nucleotides 484–502 and 1640–1620; huVEGF (M32977), nucleotides 358–379 and 962–980; mouse actin (M12866), nucleotides 244–263 and 973–954. Quantitative RT-PCR—Total RNA was isolated from frozen tissues and reverse-transcribed in 20-μl volumes using RNase H− reverse transcriptase (SuperScript II, Life Technologies, Inc.) with 1 μg (N), random primer (New England Biolabs). Aliquots (2 μl) of the reverse transcription products were used for quantitative PCR in the LightCycler™ PCR and Detection System using the FastStart DNA Master SYBR Green I kit (both from Roche Molecular Biochemicals) as described by the manufacturer. The following sense and antisense primers were used (GenBank™ accession numbers are in parentheses): muAng-2 (AF4326), nucleotides 484–502 and 1640–1620; huVEGF (M32977), nucleotides 358–379 and 962–980; mouse actin (M12866), nucleotides 244–263 and 973–954.

RESULTS

Leptin Induces Angiopoietin-2 and Apoptosis in Adipose Tissues—To determine the effects of leptin on the expression of angiogenic and angiostatic factors in adult adipose tissue, we injected murine leptin (0.1–5 μg/g, intraperitoneal) at times 0

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and 9 h into 8–10-week-old obese (C57Bl-ob/ob) female mice, lacking endogenous leptin production. A noticeable weight loss was observed after 48 h in mice receiving $231\ mg/g$ leptin (average weight 62.7 ± 1.0 g versus 65.4 ± 0.5 g, n = 6). Total RNA was isolated from subcutaneous adipose tissues at 0, 24, and 48 h after the first leptin dose, and the levels of Ang-1, Ang-2, and VEGF mRNA were evaluated by RNA blotting. Ang-1 mRNA was below the limit of detection of this analysis both before and after leptin treatment (data not shown). A significant induction of Ang-2 was observed in the leptin-treated (5 µg/g) animals. In contrast, the level of VEGF mRNA was not increased after 24 h and was only slightly elevated at 48 h (Fig. 1A). Tissue Ang-2 was analyzed by immunoblotting at 48 h post-leptin injection. No Ang-2 was detected by this method at time 0 or in tissues of mice injected with low dose (0.1 µg/g) leptin, whereas Ang-2 was induced in mice treated with 2–5 µg/g of leptin (Fig. 1B). These results demonstrated that leptin treatment induced Ang-2 in adipose tissues of C57Bl-ob/ob mice in vivo. The lack of parallel induction of VEGF by leptin indicated that the overall effect of leptin treatment on the adipose vasculature was angiostatic and not angiogenic.

It was previously reported that intracerebroventricular injection of leptin for 5 days induces significant apoptosis in rat adipose tissues (11). We found by TUNEL staining an extensive increase in the number of apoptotic nuclei as soon as 24 h after intraperitoneal injection of leptin with further increases at 48 h (Fig. 2). Apoptotic nuclei were seen particularly in endothelial cells, identified by their elongated shape and location around erythrocyte-containing microvessels (Fig. 3).

**Leptin-deficient Mice Have a Reduced Adipose Ang-2**—We then compared basal Ang-2 mRNA levels in wild-type mice, ob/ob mice, and C57Bl-db/db mice lacking the long-form leptin receptor (OB-Rb). Quantitative RT-PCR revealed that Ang-2 mRNA was expressed in the adipose tissue of all three mouse strains, indicating that leptin was not essential for Ang-2 expression (Fig. 4). However, basal Ang-2 mRNA was significantly lower in adipose tissues of ob/ob mice, suggesting that endogenous leptin plays an important role in inducing Ang-2 mRNA expression in adipose tissues. C57Bl-db/db mice have a constitutively elevated level of endogenous leptin, and yet their adipose Ang-2 was significantly lower compared with
that of wild-type mice. This result indicates that leptin induces Ang-2 by acting through OB-Rb (Fig. 4). Although OB-Rb is expressed abundantly in the hypothalamus, it is also expressed in some other cell types including adipocytes (4). Therefore, induction of Ang-2 by leptin may be regulated either centrally or locally.

**Leptin-induces Ang-2 and No VEGF in Cultured Adipocytes**—To test whether leptin can induce Ang-2 expression by acting directly on adipocytes, in vitro experiments were performed using 3T3-F442A cells. Upon transfer to medium containing fetal bovine serum, these cells differentiate into adipocytes (24, 27, 28). Leptin (1 μg/ml) induced Ang-2 mRNA expression in differentiated 3T3-F442A adipocytes but not in pre-adipocytes, as determined by RNA blot analysis (Fig. 5). This observation indicated that Ang-2 was induced by leptin in the adipose tissue in an autocrine manner. Several other cell lines were tested for Ang-2 induction by leptin. Although many of them expressed a low basal level of Ang-2, induction by leptin occurred only in adipocytes (data not shown).

We then studied the effect of leptin on VEGF mRNA induction in cultured adipocytes. Basal levels of VEGF mRNA were detected in cultured pre-adipocytes, but expression was reduced following their differentiation into adipocytes. No induction of VEGF was observed in leptin-treated adipocytes in culture. In contrast, leptin induced VEGF mRNA expression in pre-adipocytes (Fig. 5).

The kinetics and the precise levels of Ang-2 and VEGF mRNA induction in cultured adipocytes and pre-adipocytes were further measured by quantitative RT-PCR. Significant induction of Ang-2 mRNA was observed in adipocytes at 10 h, and expression was even higher at 24 h (Fig. 6). In contrast, no induction of VEGF mRNA was seen in leptin-treated adipocytes. The induction pattern was reversed in pre-adipocytes. Leptin-induced VEGF mRNA expression was significantly increased at 48 h, whereas no induction of Ang-2 was observed. The somewhat delayed kinetics of VEGF mRNA induction suggests an indirect effect of leptin on VEGF gene expression (Fig. 7).

**DISCUSSION**

Serving as an energy depot, the adipose tissue is endowed with the unique ability to expand and contract throughout adult life. This role requires the concomitant adaptation of the adipose microvasculature. In this study, we investigated the possible role of leptin on adipose microvasculature. Adipose tissues consist of several cell types, including adipocytes and fibroblasts. Our studies with these isolated cell types showed that leptin is capable of inducing both Ang-2 and VEGF in vitro in a cell-specific manner (Figs. 5–7). Indeed, induction of VEGF by leptin may account for its reported angiogenic activity in several model systems (22, 23). However, in adipose tissues of ob/ob mice, leptin profoundly induced Ang-2 without a concomitant induction of VEGF, thereby providing a strong angiostatic rather than angiogenic signal in vivo (Fig. 1). Such a process may contribute to the ablation of all adipose tissues reported upon over-expression of leptin in rats (5).

Ablation of adipose tissue is mediated at least in part by leptin-induced apoptosis, as reported by Qian et al. (11). Our data support this mechanism and further show that DNA nicking occurred mainly in the nuclei of endothelial cells (Fig. 3). Induction of Ang-2 by leptin in adipocytes and lack of VEGF may have triggered apoptosis in the endothelial cells. A similar role has been assigned to Ang-2 in the regression of ovarian follicles as well as tumor blood vessels (14, 20, 29).

The strong induction of Ang-2 by leptin in adipose tissues and in cultured adipocytes adds these cells to the rather limited number of normal adult cell types that express Ang-2. Leptin appears to be a very potent inducer of Ang-2 as compared with the previously reported agents, VEGF, bFGF, hypoxia, and tumor necrosis factor-α (19, 30, 31). Recently, the induction of genes following leptin administration to ob/ob mice was studied using oligonucleotide microarrays. Surprisingly, Ang-2 induction was not observed by this method (32). Our results,
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Although this is the first report of Ang-2 expression in adipocytes, high level expression of VEGF was found in omental adipocytes (33), providing a rationale for its well established in vivo role in adipose tissues. This induction provides a novel regulatory pathway, linking leptin to vascular homeostasis in adipose tissue. In addition to its central effects, several peripheral effects of leptin were reported, but their mechanisms have not all been elucidated (10, 22, 23, 35, 36). Induction of Ang-2 by leptin in cultured adipocytes provides convincing evidence that leptin also acts directly on peripheral targets. Such autocrine induction provides a novel regulatory pathway, linking leptin to vascular homeostasis in adipose tissue.

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FIG. 6. Quantitative RT-PCR of leptin-induced Ang-2 in cultured adipocytes. Cultures of differentiated mouse 3T3-F442A adipocytes were induced with leptin (1 μg/ml). Total RNA was extracted at different time points and subjected to quantitative RT-PCR with probes to mouse Ang-2 and actin (n = 6). Significant induction of Ang-2 mRNA (5.2 ± 0.8-fold, p < 0.02, n = 5) was observed in adipocytes at 10 h and was even higher (84 ± 4.4-fold, n = 2) at 24 h. Inset, agarose gel electrophoresis of the PCR products at cycle 30.

FIG. 7. Quantitative RT-PCR of leptin-induced VEGF in cultured pre-adipocytes. Cultures of undifferentiated mouse 3T3-F442A cells were induced with leptin (1 μg/ml). Total RNA was extracted at different time points and subjected to quantitative RT-PCR with probes to mouse VEGF and actin. Significant induction was noticed only at 48 h (n = 6). Inset, agarose gel electrophoresis of the PCR products at cycles 23 and 30.