Free Leptin Is Increased in Normal Pregnancy and Further Increased in Preeclampsia

Roberto J. Teppa, Roberta B. Ness, William R. Crombleholme, and James M. Roberts

We measured bound and free leptin levels in preeclamptic and matched normal pregnant and never-pregnant women to determine whether the free component of leptin is increased during pregnancy and further increased in preeclampsia. Two milliliters of serum was obtained from 18 normal and 18 preeclamptic patients matched by pre-pregnancy body mass index (BMI), and from 18 never-pregnant women matched by BMI with the pregnant groups. The sample was subjected to gel filtration using Sephadex G-100. Radioimmunoassay (RIA) was performed on all fractions, and the proportions of bound and free leptin were determined by analyzing the areas under the curve of the chromatographic profile. The total maternal serum leptin concentration was significantly higher in normal pregnancy compared with the nonpregnant state and was further increased in preeclampsia (33.8 ± 4.1 *v* 15.2 ± 1.8 ng/mL, *P* = .002, and 48.1 ± 5.6 ng/mL, *P* = .02, respectively). Free leptin was increased in normal pregnant compared with never-pregnant women (25.9 ± 4.1 *v* 11.0 ± 2.0 ng/mL, respectively, *P* = .01), while the increase of total leptin in preeclampsia was exclusively in the free fraction that was significantly higher versus the normal pregnant group (41.8 ± 5.6 *v* 25.9 ± 4.1 ng/mL, respectively, *P* = .01). The bound leptin fraction, by contrast, was significantly increased in the normal pregnant group compared with the preeclamptic group and the never-pregnant group (7.9 ± 0.56 *v* 6.2 ± 0.36 and 4.1 ± 0.36 ng/mL, respectively, *P* = .009 and *P* = <.0001). In conclusion, the free leptin concentration increases in normal pregnancy and is further increased in preeclampsia. This supports the hypothesis that biologically active leptin is elevated in normal pregnancy and is increased more in women with preeclampsia.

LEPTIN, the product of the ob gene, is produced primarily by adipose tissue[1-4] but also by the human placenta.[5-7] Since many of the metabolic alterations of preeclampsia (increased free fatty acids and triglycerides)[8-10] are consistent with the known effects of leptin (increased lipolysis),[11-13] we previously measured leptin levels and reported that total plasma leptin is increased in preeclamptic compared with normal pregnant women.[14] Leptin circulates in a bound form and a free form,[15,16] and as with most hormones, it is the free fraction which is likely bioactive. Free leptin is the component altered by perturbations known to alter the total leptin concentration (eg, feeding and fasting).[16]

Recent data in experimental animals have suggested alternative explanations for the increase of leptin during pregnancy which may or may not be relevant to humans. In mice, leptin measured by radioimmunoassay (RIA) increased 20- to 40-fold in pregnancy. There is no mRNA for leptin in the murine placenta, and it appears that the explanation for the increase is a placentally derived soluble receptor.[17] The vast majority of leptin in pregnant mice is bound to this protein, and it is postulated that this reduces the renal excretion of leptin, a major route of leptin disposal.[18-20] The biological activity of this bound leptin has not been established.

To ascertain that it is the free component of leptin which is increased in human pregnancy and further increased with preeclampsia, we measured bound and free leptin levels in preeclamptic, normal pregnant, and never-pregnant women.

SUBJECTS AND METHODS

Subjects

Serum samples were obtained as part of an ongoing study of preeclampsia that has been approved by the Magee-Womens Hospital Institutional Review Board. Samples were obtained at the time of admission to labor and delivery, processed within 2 hours, and stored at −70°C. Most pregnant women were not fasting when the samples were obtained. Samples were identified from 18 nulliparous pregnant women with preeclampsia and from 18 normal nulliparous pregnant women matched pairwise by pre-pregnancy body mass index (BMI) with the preeclamptic group. Additionally, we obtained fasting samples (>8 hours) from 18 never-pregnant women matched by BMI with the pregnant groups. We excluded from this nonpregnant group any women with a medical history of liver disease, coagulation disorder, or use of steroids or any medication to lose weight by the time of the study.

We included this never-pregnant group for the purpose of obtaining a baseline with which to compare our data in the pregnant state. Examining never-pregnant women was especially important because of the prolonged effect of pregnancy on the leptin concentration, with changing leptin levels present up to 2 years postpartum.[21]

Because of the reported variability of leptin with tobacco usage,[22-25] all selected women reported that they did not smoke.

Preeclampsia was defined using the criteria of hypertension, proteinuria, hyperuricemia, and reversal of hypertension and proteinuria after pregnancy.[26] Hypertension was defined as an increase of 30 mm Hg systolic or 15 mm Hg diastolic blood pressure as compared with values obtained before 20 weeks' gestation, or an absolute blood pressure of 140/90 mm Hg or higher after 20 weeks' gestation if earlier blood pressure values were unknown. Among the 18 preeclamptic patients, all had a blood pressure greater than 140 mm Hg systolic or 90 mm Hg diastolic except 1 woman who, despite the diagnostic blood pressure increase of 30 mm Hg systolic and 15 mm Hg diastolic, achieved a blood pressure of only 138/88 mm Hg. This particular patient had total and free leptin concentrations above the median of the group (54 ng/mL total and 50 ng/mL free leptin). Proteinuria was defined as 300 mg/24 h collection, or more than 2+ on a voided or 1+ on a catheterized random urine specimen.

Hyperuricemia was defined as more than 1 standard deviation above the usual value for the gestational age at which the sample was obtained

From the Magee-Womens Research Institute and Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh, Pittsburgh, PA.

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Address reprint requests to Roberto J. Teppa, MD, Magee-Womens Research Institute, 204 Craft Ave, Room 620, Pittsburgh, PA 15213.

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(at term >5.5 mmol/L). All abnormalities returned to normal by 12 weeks postpartum.

Gel Exclusion and RIA: Determination of Total, Bound, and Free Leptin in Human Serum

A column (length, 50 cm; ID, 14.56 mm) was packed with Sephadex G-100 (total vol, 80 cm^3). All separations were performed at 4°C using 25 mmol/L phosphate-buffered saline as the eluate (pH 7.4 containing 0.01% sodium azide) at a flow rate of 460 μL/min. The column was loaded with 2-mL serum samples, and 1.4-mL fractions were collected. To determine void and bed volume, a single 900-μL aliquot of the serum sample from a control pregnant patient was incubated with [^{125}I]leptin (561.8 dpm; machine efficiency, 75%) for 24 hours at 4°C and then eluted. The bound fraction was indicated by radioactivity eluting in the void volume, whereas the free fraction was the radioactivity eluting in the [^{125}I]leptin region. The sum of radioactivity eluting in the 2 peaks was calculated, and recovery was determined as the percentage of added radioactive leptin eluted (73.1% ± 1.0%, n = 12). No appreciable dissociation (<10%) of bound [^{125}I]leptin was observed when peak 1 was rechromatographed at 4°C. RIA was performed on all fractions eluting between the void and bed volumes using a commercially available [^{125}I]human leptin RIA kit (Linco Research, St. Charles, MO). The detection limit was 0.5 ng/mL. The mean within-assay coefficient of variation was 2.8% and between-assay coefficient of variation 9%. The proportions of bound and free leptin were determined by analyzing the areas under the curve of the chromatographic profile. Absolute levels of bound and free leptin were then calculated by multiplying the percent bound and percent free leptin by the total leptin concentration.

A standard curve was generated with human leptin and fitted with an iterative non-linear curve-fitting program using Igor Pro 3.01 (Wave Metrics, Lake Oswego, OR) software.

Two pools of serum from preeclamptic women (n = 12) and normal pregnant women (n = 12) from a subset of the initial groups (based on sample availability) were also prepared. Another pool was prepared from the serum of never-pregnant women (n = 18). Two-milliliter aliquots from each pool were subjected to gel filtration, and RIA was performed on all fractions collected to determine the concentration of bound and free leptin. Two-milliliter aliquots from these same pools were incubated with [^{125}I]leptin (specific activity, 135 μCi/μg) for 24 hours at 4°C (750 dpm). Finally, 2 other aliquots were dialyzed for 72 hours at 4°C (25,000 MWCO cellulose ester tubing; Spectrum Medical Industries, Houston, TX). These samples were then incubated with 750 dpm [^{125}I]leptin. The dialyzed and undialyzed aliquots were subjected to gel filtration to determine the distribution of [^{125}I]leptin.

Statistical Analysis

The data are expressed as the mean ± SEM. For statistical analysis, we performed factor ANOVA with post hoc testing (Fisher's protected least-significant difference) and Student's *t* test (unpaired) using StatView 4.5 (Abacus Concepts, Berkeley, CA). Statistical significance was accepted at a *P* level less than .05 for all comparisons. Correlation coefficients were used to express relationships between continuous variables. Nonparametric analysis was used if the data were not normally distributed.

RESULTS

Group Characteristics

The demographic and clinical characteristics of the preeclamptic and matched normal pregnant and never-pregnant women are shown in Table 1. As expected from the matching criteria used in this study, there was no significant difference between groups with respect to either the pre-pregnancy BMI or BMI at sampling. The mean pre-pregnancy BMI and BMI at sampling of the preeclamptic group were 26.4 ± 1.1 and 32.9 ± 1.1 kg/m^2, respectively, while the mean pre-pregnancy BMI and BMI at sampling of the normal group were 25.6 ± 1.0 and 31.5 ± 1.0 kg/m^2, respectively. The mean BMI of the never-pregnant group was 25.8 ± 1.0 kg/m^2. There was a significant difference in gestational age at sampling between preeclamptic and normal pregnant patients (36.6 ± 0.4 *v* 38.2 ± 0.3 weeks). Previous studies have not found differences in leptin across this gestational age range.[5,27] Similarly, in our study, gestational age was not correlated with leptin concentrations (total, free, or bound) in the total group (r^2 = .05) or either experimental group (r^2 = .002 for the preeclamptics and r^2 = .024 for the control group). Maternal age was also greater for preeclamptic women compared with the normal pregnant group (26.5 ± 1.5 *v* 21.1 ± 0.8 years, *P* = .003). Leptin concentrations have not been found to be influenced by age across this narrow age range,[28] and there was not a significant relationship in this study for total, free, or bound leptin in the total pregnant group (total r^2 = .002, free r^2 = .005, and bound r^2 = .04) in cases (total r^2 = .1, free r^2 = .1, and bound $r^2 = 4.8 \times 10^{-4}$) or in controls (total r^2 = .1, free r^2 = .1, and bound r^2 = .009).

Table 1. Comparative Group Characteristics (mean ± SEM) of Nonsmoking Women With Preeclampsia (n = 18), Normal Pregnant Women (n = 8), and Never-Pregnant Women Matched 1:1 on Pre-Pregnancy BMI or BMI

Characteristic	Preeclamptic (n = 18)	Normal (n = 18)	Never-Pregnant (n = 18)
Pre-pregnancy BMI or BMI (kg/m^2)	26.4 ± 1.1	25.6 ± 1.0	25.8 ± 1.0
BMI at sampling (kg/m^2)	32.9 ± 1.1	31.5 ± 1.0	
Gestational age at sampling (wk)*	36.6 ± 0.4	38.2 ± 0.3	
Maternal age (yr)*	26.5 ± 1.5	21.1 ± 0.8	24.5 ± 1.2
SBP before 20 weeks' gestation (mm Hg)	113.6 ± 2.0	114.2 ± 1.6	
DBP before 20 weeks' gestation (mm Hg)	70.1 ± 1.4	70.0 ± 1.4	
SBP at delivery (mm Hg)*	149.7 ± 2.7	125.0 ± 2.0	
DBP at delivery (mm Hg)*	93.8 ± 1.5	76.0 ± 1.2	
Maternal creatinine predelivery (mg/dL)	0.6 ± 0.04	0.5 ± 0.03	
Newborn infant birth weight (g)*	2,630.0 ± 130.0	3,274.5 ± 105.1	

*P < .05 for preeclamptics *v* normals.

As required by the classification criteria used in this study, significant differences between preeclamptic and normal groups were noted for both systolic and diastolic predelivery blood pressure, but not for the blood pressure reading before 20 weeks' gestation.

There was no significant difference between the pregnant groups with respect to maternal predelivery creatinine concentrations (0.6 ± 0.04 mg/dL for the preeclamptics and 0.5 ± 0.03 mg/dL for the control group). There was also no correlation

between the serum leptin concentration and maternal creatinine concentration in these women (all subjects, total $R^2 = .005$, free $r^2 = .009$, bound $r^2 = .07$; cases, total $r^2 = .01$, free $r^2 = .01$; bound $r^2 = .02$; and controls, total $r^2 = .002$, free $r^2 = .005$, bound $r^2 = .02$).

The difference in birth weight between infants of preeclamptics (2,630 ± 130.0 g) and controls (3,274 ± 105.1 g) was statistically significant ($P = .0005$). As expected, the number of infants with a birth weight percentile for gestational age less than 10% was higher in the preeclamptic group (4 of 18) than in the gestational age–matched normal group (0 of 18).

Leptin Concentration

The maternal total serum leptin concentration was measured prior to delivery in 18 pairs of preeclamptic and matched normal pregnant women. Serum leptin was also measured in the serum of 18 never-pregnant women. Total serum leptin was significantly higher in the preeclamptic group (48.1 ± 5.6 ng/mL) compared with the normal group (33.8 ± 4.1 ng/mL, $P = .02$) and the never-pregnant group (15.2 ± 1.8 ng/mL, $P \leq .0001$). Total serum leptin was also significantly higher in the normal pregnant group compared with the never-pregnant women ($P = .002$; Fig 1). After chromatographic separation (Fig 2), the free leptin fraction was also significantly higher in the preeclamptic group (41.8 ± 5.6 ng/mL) compared with the normal group (25.9 ± 4.1 ng/mL, $P = .01$) and the never-pregnant group (11.0 ± 2.0 ng/mL, $P \leq .0001$; Fig 3). The free leptin fraction was significantly higher in the normal pregnant group compared with the never-pregnant group ($P = .01$). By contrast, the normal pregnant group had a significantly higher bound leptin fraction (7.9 ± 0.5 ng/mL) compared with the preeclamptic group (6.2 ± 0.3 ng/mL, $P = .009$) and the never-pregnant group (4.1 ± 0.3 ng/mL, $P \leq .0001$). The bound fraction was also higher in preeclamptics compared with the never-pregnant group ($P = .001$; Fig 4). Because of the difference in bound leptin, we examined the binding capacity of pooled serum from 12 preeclamptic women, 12 normal pregnant women, and 18 never-pregnant women. The binding of [^{125}I]leptin was tested before and after dialysis of the 3 pools (Fig 5). After removal of endogenous leptin, the binding

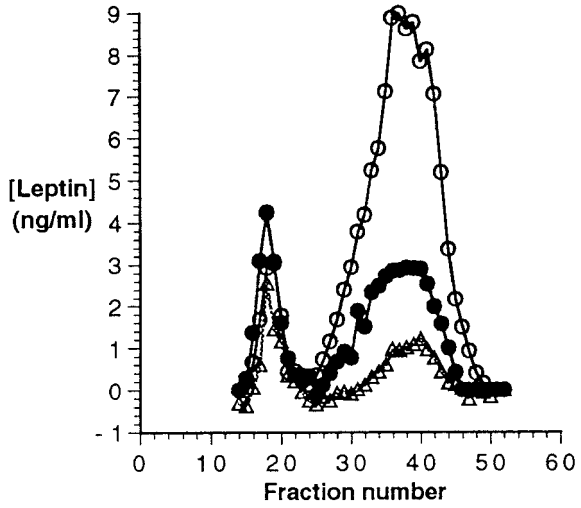

Fig 2. Chromatographic profile of immunoreactive leptin in pooled sera of normal (●), preeclamptic (○), and never-pregnant women (△). Sera pooled from 12 preeclamptic, 12 normal pregnant, and 18 never-pregnant women were subjected to gel-exclusion chromatography (Sephadex G-100) and fractions were analyzed for leptin by RIA. Total leptin in preeclamptic, normal, and never-pregnant women was 65, 30, and 5 ng/mL, respectively, while free leptin was 59 ng/mL (92%) in preeclamptic, 20 ng/mL (68%) in normal pregnant, and 3 ng/mL (62%) in never-pregnant women.

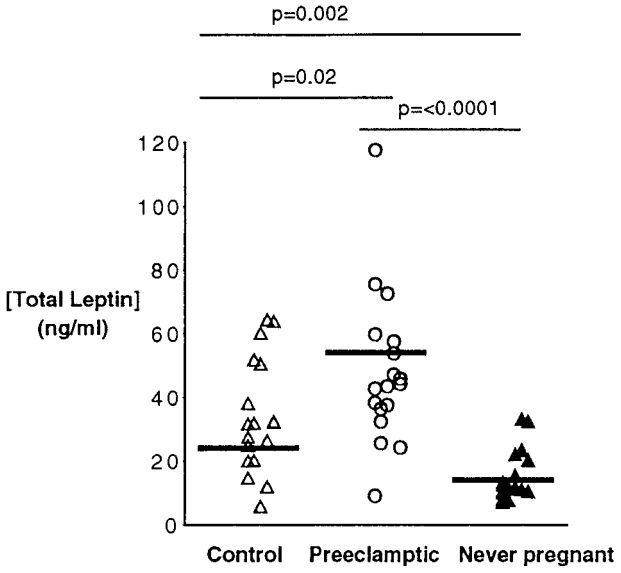

Fig 1. Total serum leptin concentration in normal (△), preeclamptic (○), and never-pregnant women (▲). Leptin was measured by RIA prior to delivery in 18 pairs of preeclamptic and matched normal pregnant women, and in matched never-pregnant women. Preeclampsia ($P = .02$) *v* respective normal pregnancy or never-pregnant group, ($P \leq .0001$) (ANOVA with post hoc testing).

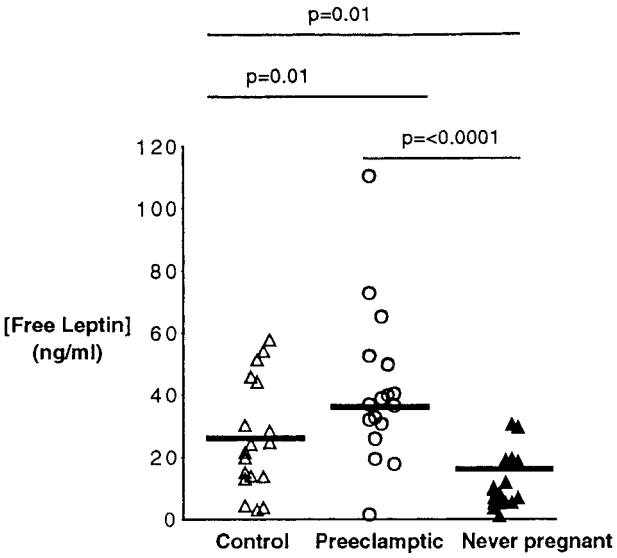

Fig 3. Free leptin concentration in normal (△), preeclamptic (○), and never-pregnant women (▲). Leptin was measured by RIA on fractions collected through gel filtration of serum from preeclamptic, normal, and never-pregnant women. Preeclampsia ($P = .01$) *v* respective normal pregnancy or never-pregnant group, $P \leq .0001$ (ANOVA with post hoc testing). Bars indicate the median.

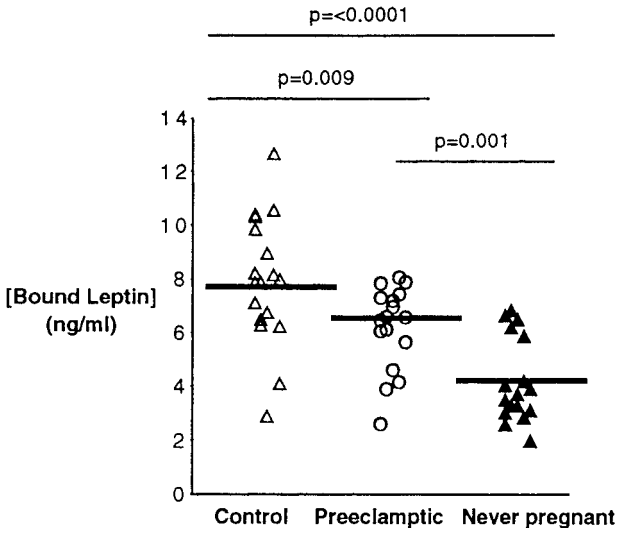

Fig 4. Bound leptin concentration in normal (△), preeclamptic (○), and never-pregnant women (▲). Leptin was measured by RIA on fractions collected through gel filtration of serum from preeclamptic, normal pregnant, and never-pregnant women. Preeclampsia ($P = .009$) *v* respective normal pregnancy or never-pregnant group, $P = .001$ (ANOVA with post hoc testing). Bars indicate the median.

capacities were similar in serum from preeclamptic, normal, and never-pregnant patients (Fig 5).

As previously reported, the BMI and total leptin were closely correlated in never-pregnant women ($r^2 = .2$, $P = .02$). A similar correlation was present for free leptin ($r^2 = .3$, $P = .01$). The pre-pregnancy BMI and BMI at sampling were positively correlated with total serum leptin ($r^2 = .4$, $P = .006$ and $r^2 = .2$, $P = .002$, respectively) and free leptin ($r^2 = .4$, $P = .005$ and $r^2 = .2$, $P = .002$, respectively) in the preeclamptic group.

The BMI did not correlate with bound leptin in preeclamptic or control subjects, and correlated negatively in the never-pregnant subjects ($r^2 = .2$, $P = .05$). There was no correlation between serum leptin and blood pressure in preeclampsia or normal pregnancy. There also was no correlation between maternal leptin concentrations (total, free, or bound) and neonatal birth weight in the total patient sample (total $r^2 = .15$, free $r^2 = .15$, and bound $r^2 = .001$) or in the individual groups (total $r^2 = .08$, free $r^2 = .05$, and bound $r^2 = .16$) for the preeclamptics (total $r^2 = .09$, free $r^2 = .08$, and bound $r^2 = .005$) and for controls.

From the total of 18 patients, there were 8 patients for whom we had information regarding the time of the last meal within each pregnant group. For each pregnant group, 3 had been fasting more than 8 hours and 5 less than 8 hours. The mean time from the last meal between groups was not significantly different (preeclamptic *v* control, 6.1 ± 2.1 *v* 8.2 ± 2.1 hours, $P = .5$). All subjects from the never-pregnant group fasted longer than 8 hours.

DISCUSSION

Using patients who were not tested previously, we confirmed our prior observation that the total maternal plasma leptin concentration is significantly increased in women with preeclampsia compared with normal pregnant women.[14] In addition, we now report that the increase in total leptin during preeclampsia and normal pregnancy is secondary to an increase of the free fraction. Thus, it would appear that the increase of serum leptin in preeclampsia is biologically relevant. Similarly, there is an increase of biologically active leptin with normal pregnancy.

The usual source of leptin in the nonpregnant state is adipose tissue, and thus, the leptin concentration correlates with the BMI in humans. However, in pregnancy, the BMI does not accurately reflect fat accrual, since the fetus, placenta, amniotic fluid, increased plasma volume, and a variable degree of extravascular fluid accumulation also increase maternal weight. The contribution of extravascular fluid is especially problematic in women with preeclampsia, as fluid retention is one of the

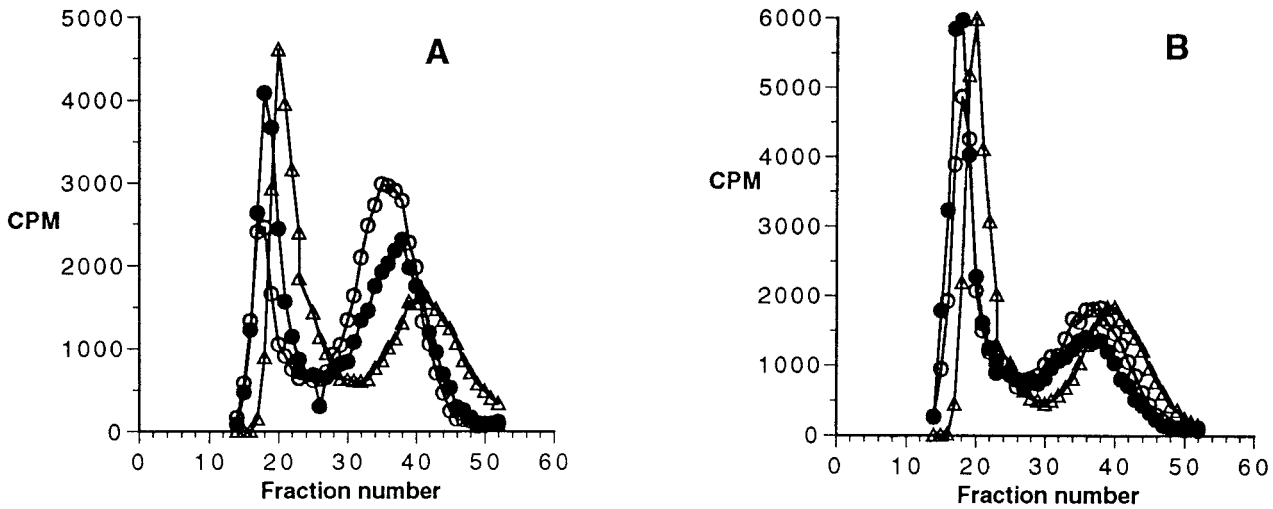

Fig 5. Chromatographic profile of [^{125}I]leptin added to pooled serum before and after dialysis. Serum was pooled from 12 preeclamptic (○), 12 normal pregnant (●), and 18 never-pregnant women (△). [^{125}I]leptin was added to 1 aliquot before (A) and 1 aliquot after (B) the removal of endogenous leptin from the carrier protein by dialysis. Although [^{125}I]leptin binding (first peak) was different prior to dialysis (A), the difference was largely eliminated by the removal of endogenous leptin (B).

cardinal features of this disorder. Thus, to control for the effect of adiposity on the maternal leptin concentration, we chose to match subjects based on the pre-pregnant BMI. Nonetheless, the BMI was not different in the 2 groups at the time of sampling. On the basis of our matched study design, it seems unlikely that the increased leptin concentration in preeclampsia relates to differences in maternal adiposity during pregnancy.

We found that the gestational age at sampling was lower in women with preeclampsia. Previous studies have not found differences in leptin across this gestational age range.[5,27] Additionally, gestational age did not correlate with leptin concentrations in the total population or in either group. One preeclamptic woman had much higher total and free leptin values than the rest of the group (Figs 1 and 3). Because the data were analyzed nonparametrically, this did not influence the analysis. We could not find a ready explanation for this high concentration. It was not explained by the BMI or creatinine level (33.8 kg/m^2 and 0.6 mg/dL), which were not strikingly different versus the mean values for all preeclamptic women. It also did not match with the severity of disease as indicated by blood pressure (119/80 mm Hg at $<$20 weeks of gestation and 141/95 mm Hg at the time of delivery). In a probably unrelated development, this patient gave birth to a newborn with myelomeningocele who died at the third week postdelivery.

We did not have information on the fasting state of 10 patients within each pregnant group. In patients for whom we had meal information (8 in each group), the number of patients who fasted longer than 8 hours and the mean time from the last meal were not different in cases and controls. In studies examining glucose loading, there was a 16% peak change in leptin after 75 g oral glucose that was only observed in obese women.[29] This is much less than the 2-fold change between normal pregnant and never-pregnant patients and the 50% further increase with preeclampsia. Furthermore, in fasting studies, leptin did not decrease until 12 hours after the onset of fasting.[30] It appears that the postprandial status does not explain the differences in leptin concentrations between preeclamptic and normal pregnant women.

Although this finding supports our initial hypothesis that biologically active leptin is increased in women with preeclampsia, caution is necessary when comparing blood concentrations of substances in preeclamptic and normal pregnant women.[31] First, impaired renal function is a pathophysiologic component of preeclampsia, and the measured increase in the leptin concentration may reflect reduced renal clearance. There is evidence that the kidneys are important for the elimination of leptin from the circulation, which is mediated in part by glomerular filtration.[18,19] Since the maternal plasma creatinine concentration was not significantly different between the 2 pregnant groups studied, it appears unlikely that differences in glomerular filtration could account for our observations. We also did not find a correlation for free or bound leptin with creatinine in any group. Second, there is the concern that the observed increase in leptin could be secondary to the hemoconcentration that is present in preeclampsia.[32] In our previous study, we did not find a correlation with the hematocrit.[14] Furthermore, we would expect that hemoconcentration would increase both leptin fractions (bound and free) proportionally, which was not the case in this study.

Another possible explanation for the increase of free leptin in preeclampsia would be a recompartmentalization to the free compartment in association with a marked reduction in leptin binding protein. This is suggested qualitatively by our finding of a slightly reduced concentration of bound leptin in preeclamptic women. Quantitatively, this is not a viable explanation, as the increase in free leptin far exceeds the slightly lower bound concentration. In addition, the similarity in the binding profile of radioactive leptin after removal of free leptin by dialysis does not support a large difference in the concentration of leptin binding proteins or a substantial difference in affinity. Likewise, there does not appear to be a substantial change in the leptin binding capacity between the pregnant and nonpregnant states. Thus, our data would suggest an increase in leptin production in preeclamptic women. This conclusion is supported by the finding that leptin mRNA is increased in the placenta of women with preeclampsia.[6] The same group found that exposing a trophoblast cell line to hypoxic conditions, as is posited to occur in preeclampsia, resulted in an increase in leptin production.

These findings raise the possibility that leptin production may increase in preeclampsia as an adaptive response by the fetus or placenta to reduced perfusion. Such a response might serve to satisfy fetal metabolic demands. An increase in the delivery of leptin from the placenta to the mother with a subsequent increase in lipolysis would result in increased maternal circulating free fatty acids and glucose availability for the fetus. The increased availability of substrate could compensate for the reduction in placental perfusion and result in normal substrate delivery to the fetus. This would increase the fetal body mass and subsequently increase fetal leptin. Since fetal adiposity is an important regulator of fetal leptin,[33] maternal and neonatal leptin concentrations would be predicted to correlate in preeclamptics, an observation we previously reported, although this is not the case in normal pregnancy.[14] We posit that some women are not able to tolerate the metabolic alterations induced by the increased leptin and will develop preeclampsia.

Preeclamptic women are known to have increased sympathetic activity,[34] and there is evidence suggesting that leptin increases the sympathetic outflow.[35,36] Further evidence that leptin may play a role in the pathophysiology of preeclampsia is the increase in blood pressure through chronic (over 1 week) infusion in rats.[37] Blood pressure can be increased with leptin infusion, perhaps related to the effects on the sympathetic nervous system mediated by the renin-angiotensin-aldosterone system.[38,39]

There is abundant evidence with regard to endothelial dysfunction in women with preeclampsia which could be produced by oxidative stress. Leptin also has been shown to produce oxidative stress in human endothelial cells through the accumulation of reactive oxygen species.[40]

In conclusion, the present study demonstrates that the increase in leptin during normal pregnancy and further increase in preeclampsia are due to an increase of the presumably biologically active free leptin fraction.

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