Regulation of Leptin Release by Troglitazone in Human Adipose Tissue

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In pieces of human subcutaneous adipose tissue incubated in primary culture for 48 hours, the release of leptin was stimulated by 50% in the presence of 3.3 μmol/L troglitazone. Insulin (0.1 nmol/L) and dexamethasone (200 nmol/L) stimulated leptin release by 30% and 300%, respectively. Troglitazone in combination with either insulin or dexamethasone had no effect on leptin release. Instead, troglitazone inhibited leptin release in the presence of both dexamethasone and insulin. The stimulatory effect of troglitazone on leptin release was also mimicked by 1 μmol/L 15-deoxy-$\Delta^{12\text{-}14}$prostaglandin J_2 (dPGJ_2). However, if the concentration of dPGJ_2 was increased to 10 μmol/L in the presence of dexamethasone, there was a decrease in leptin release, as well as of lactate formation and lipolysis. These data indicate that both stimulatory and inhibitory effects of troglitazone and dPGJ_2 can be seen on leptin release by human adipose tissue.

TROGLITAZONE IS A member of the thiazolidinedione family of drugs that are effective oral antihyperglycemic agents.[1] In poorly controlled non–insulin-dependent diabetes mellitus (NIDDM) Japanese patients (average body mass index [BMI], 25) who were treated for 12 weeks with 400 mg/d of troglitazone, there were significant decreases in blood glucose, insulin, hemoglobin A1c, and leptin.[2] However, this improvement was accompanied by an increase in hunger, as well as in the BMI.[2] In obese Zucker rats, the administration of troglitazone for 15 days normalized blood glucose and insulin, but the weight of the epididymal fat pads increased by almost 9%.[3] The level of leptin mRNA in the epididymal fat was markedly diminished along with the number of large adipocytes, indicating that the increase in fat weight was due to a marked increase in the number of small fat cells.[3] Zhang et al[4] treated obese Zucker rats for 28 days with a thiazolidinedione and found a 39% increase in body weight, which was accompanied by a marked decrease in the level of leptin mRNA.

An increased proliferation of adipocytes in response to thiazolidinediones has also been seen in a bone marrow stromal cell line.[5] One of the major regulators of adipose cell differentiation is peroxisome proliferator-activated receptor γ (PPARγ), an adipocyte-specific member of the nuclear hormone receptor superfamily that appears to be the natural ligand for troglitazone and other thiazolidinediones.[6,7] In addition, some actions of troglitazone occur independently of adipose tissue, because hyperglycemia and elevated serum insulin in fat-free mice could be normalized by troglitazone.[8]

In isolated human adipocytes, Nolan et al[9] reported that 10 μmol/L troglitazone inhibited basal and insulin-stimulated leptin release. Similarly, De Vos et al[10] reported that in rat adipocytes incubated for 24 hours with 100 μmol/L BRL 49653, which is a thiazolidinedione, there was a marked decrease in the level of leptin mRNA. The present studies used cut pieces of human adipose tissue incubated in primary culture for 48 hours and indicated that there were both stimulatory and inhibitory effects of troglitazone on leptin release.

SUBJECTS AND METHODS

Subjects

Subcutaneous abdominal adipose tissue was obtained from 18 morbidly obese females and 1 male undergoing elective open abdominal surgery (gastric bypass) under general anesthesia after an overnight fast. The BMI of these patients ranged from 42 to 67, and the average BMI was 54. The mean age of the patients was 39 years, with a range of 19 to 60. The fasting blood glucose value averaged 103 mg/dL. One individual had a blood glucose value of 197 mg/dL, and all others had values below 115 except for 1 individual with a value of 131 mg/dL. The fasting plasma insulin averaged .12 ± .02 nmol/L (n = 17, mean ± SEM). The individual with a blood glucose of 197 mg/dL had a plasma insulin of 0.10 nmol/L. Adipose tissue was also obtained from 3 previously markedly obese females 1 year after gastric bypass surgery when they were hospitalized for abdominoplasty (BMI, 22, 33, and 33). No experiments were excluded from data analysis. The study had the approval of the local ethics committee, and all patients involved gave informed consent. The patients were fasted overnight before surgery, but had not been on any type of dietary restriction just before surgery.

Materials

Bovine serum albumin powder (Bovuminar lot K59410, containing <0.05 mol fatty acids/mol albumin) was obtained from Intergen (Purchase, NY), troglitazone (Rezulin) was a gift of Parke-Davis, (Morris Plains, NJ), and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (dPGJ_2) was obtained from Cayman Chemical (Ann Arbor, MI). Insulin and other chemicals were from Sigma Chemical (St Louis, MO). All agents were added at the start of the 48-hour incubation. Stock solutions of troglitazone and dPGJ_2 were prepared in ethanol and added in 5 μL of ethanol. The same amount of ethanol was added to control flasks.

Adipose Tissue Culture

Cut pieces of adipose tissue (~400 mg) were incubated as previously described in Dulbecco's modified Eagle's medium/Ham's F12.[11] All agents were added at the start of the incubation. Aliquots of the medium were taken at 48 hours and stored at −20°C for measurement of leptin and lipolysis. The leptin content of 20- to 50-μL aliquots of the incubation medium was determined using human leptin radioimmunoassay kits from Linco Research (St Charles, MO), as was the plasma insulin. Lipolysis was based on analysis of glycerol release into the medium.[11] Total RNA was extracted from adipose tissue at the start and at the end of the incubation.[12,13] Leptin mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were analyzed simulta-

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neously by Northern blot analysis using a ^{32}P-labeled mouse leptin cDNA probe and a human GAPDH cDNA probe. It was necessary to increase the sensitivity of the Northern blot analysis by the inclusion of ULTRAhyb (Ambion, Austin, TX) in the hybridization buffer. The radioactivity in leptin mRNA in each experiment was corrected for recovery of 18S RNA and also expressed as the ratio of cpm recovered in leptin mRNA divided by that in GAPDH mRNA.

Statistical analysis was based on Student's *t* test for the data shown in Figs 1 through 3. The statistical analysis in Table 1 was repeated measures analysis of variance, run using version 8.0 of the SPSS (Chicago, IL) "GLM" procedure on a Dell (Austin, TX) laptop with a Windows 95 platform (Microsoft, Elbgrove, CA). The data analysis was based on the percentage differences due to added agents to normalize the data, because the basal release of leptin was quite variable. Basal release of leptin over 48 hours in the absence of any added agents ranged from 30 to 318 ng/g of adipose tissue in 22 individuals. There was no significant correlation between leptin release and BMI, plasma insulin, or blood glucose for the 22 individuals (unpublished data).

RESULTS

The addition of troglitazone to pieces of human adipose tissue resulted in a stimulation of leptin release over a 48-hour incubation (Fig 1). We used a 48-hour incubation because dexamethasone, a synthetic glucocorticoid, stimulated leptin release after 48, but not 24 hours of incubation.[11] The maximal stimulation of leptin release (49%) was seen in the presence of 3.3 μmol/L troglitazone, while 1 μmol/L troglitazone increased leptin release by 33%, and both differences were significant with $P < .001$. Increasing the concentration of troglitazone to 10 μmol/L reduced the stimulation by about half indicating that approximately 3.3 μmol/L troglitazone had the maximal effect (Fig 1).

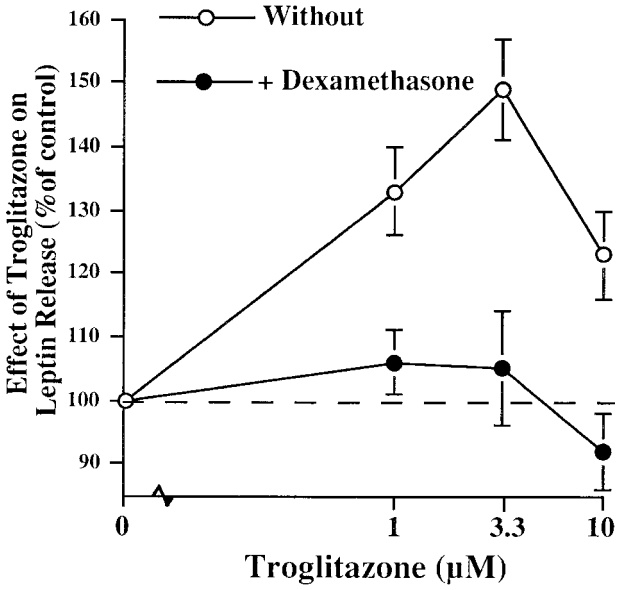

Fig 1. Troglitazone stimulates leptin release by human adipose tissue in the absence of dexamethasone. Fragments of human subcutaneous adipose tissue (~400 mg) were incubated for 48 hours in 5 mL of medium either without (○), with 200 nmol/L dexamethasone (●). The values are shown as the mean of ± SEM of the paired percentage differences due to 1, 3.3, or 10 μmol/L troglitazone using fat from 17 different individuals. The basal value for leptin release was 114 ± 17 ng/g of fat in the absence and 321 ± 26 ng/g of fat in the presence of dexamethasone.

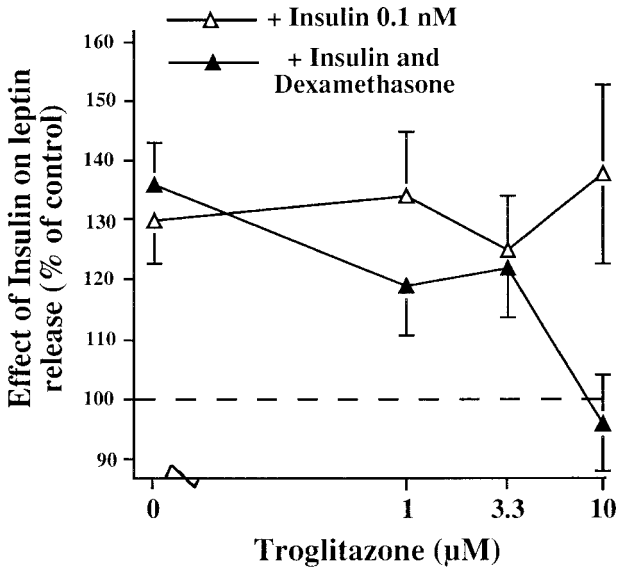

Fig 2. Troglitazone inhibits leptin release in the presence of insulin and dexamethasone. Fragments of adipose tissue (400 mg) were incubated for 48 hours in 5 mL of medium without (△) or plus 200 nmol/L dexamethasone (▲). The values are shown as the percentage change ± SEM due to insulin from the 48-hour basal value, which was 121 ± 20 ng/g of leptin in the absence and 350 ± 45 ng/g in the presence of dexamethasone with the indicated concentrations of troglitazone. The values are for adipose tissue from 14 different individuals.

In the presence of 200 nmol/L dexamethasone, basal leptin release was increased from 114 ± 17 to 321 ± 26 ng of leptin per gram of fat over 48 hours ($P < .001$) in the studies shown in Fig 1. However, there was no statistically significant effect of troglitazone on leptin release in the presence of dexamethasone (Fig 1).

We found that the leptin mRNA content of adipose tissue after a 48-hour incubation was 61% ± 10% of the initial value in the absence and 72% ± 13% in the presence of 3.3 μmol/L troglitazone (mean ± SEM of 14 experiments). The leptin mRNA content after 48 hours was 124% ± 34% of the initial value in the presence of 200 nmol/L dexamethasone and 174% ± 48% in the presence of troglitazone plus dexamethasone (mean ± SEM of 9 experiments). If the data were analyzed as the percent change due to 3.3 μmol/L troglitazone in the ratio of leptin mRNA/GAPDH mRNA, the change was still not statistically significant (−7% ± 8% in the absence and +34% ± 22% due to troglitazone in the presence of dexamethasone). These data indicate that while there was a clear effect of dexamethasone on leptin mRNA, no statistically significant effect on leptin mRNA could be seen with troglitazone.

The stimulation of leptin release by 1 μmol/L troglitazone (33%) in the absence of dexamethasone was similar to the 28% increase in leptin release seen with 0.1 nmol/L insulin (Fig 1 *v* Fig 2). The combination of insulin plus 10 μmol/L troglitazone had an effect equivalent to that of insulin alone (Fig 2). Thus, insulin or dexamethasone blunted the stimulatory effect of troglitazone on leptin release from human adipose tissue.

In the presence of 200 nmol/L dexamethasone, the percentage increase in leptin release due to 0.1 nmol/L insulin was not significantly different than that seen in the absence of dexamethasone (Fig 2). This differentiates the effect of insulin from that of troglitazone whose stimulation of leptin release was abolished in the presence of 200 nmol/L dexamethasone (Fig 1). Furthermore, troglitazone at 10 μmol/L actually blocked the stimulation of leptin release by insulin in the presence of dexamethasone (Fig 2).

It has been reported that thiazolidinediones reverse cyclic adenosine monophosphate (cAMP)-induced insulin resistance in mouse 3T3-L1 cells[14] or rat adipocytes.[15] Therefore, we examined the effects of 10 μmol/L troglitazone on leptin release and lipolysis in the presence of forskolin, which is a direct activator of adenylyl cyclase (Table 1). Forskolin (2 μmol/L) increased lipolysis by 30% to 65% over 48 hours in the absence or presence of dexamethasone. Troglitazone did not significantly affect forskolin-stimulated lipolysis either in the absence or presence of insulin (Table 1).

A 13% trend toward inhibition of leptin release in the presence of forskolin was observed ($P = .071$), and a 33% inhibition of leptin release in the presence of forskolin plus dexamethasone was reliably documented ($P = .004$, Table 1). Troglitazone (10 μmol/L) abolished the apparent inhibition of leptin release by forskolin in the absence of dexamethasone (Table 1). This was in agreement with data shown in Fig 1, in which there was stimulation of leptin release by 10 μmol/L troglitazone in the absence of forskolin.

There was no significant inhibition of leptin release by forskolin in the presence of insulin, either with ($P = .956$) or without ($P = .876$) the presence of dexamethasone. However, 10 μmol/L troglitazone inhibited leptin release by 22% in the presence of dexamethasone, forskolin, and insulin ($P = .029$, Table 1). This was in contrast to the 25% stimulation ($P = .049$) of leptin release due to 10 μmol/L troglitazone in the presence of forskolin and insulin. These data clearly indicate that under appropriate conditions troglitazone can either stimulate leptin release (the absence of insulin, forskolin, or dexamethasone or the presence of forskolin plus insulin), have no effect (the presence of forskolin or insulin), or inhibit leptin release in the presence of all 3 agents.

Prostanoids such as $dPGJ_2$ can activate PPARγ,[16,17] but are also potent inhibitors of IKB kinase.[18,19] Sinha et al[16] recently reported that in cultured 3T3-L1 mouse adipocytes, $dPGJ_2$ at concentrations in the range of 0.1 to 10 μmol/L inhibited leptin release. They also reported a similar inhibition of leptin release by 0.1 to 1 μmol/L troglitazone confirming the work of Kallen and Lazar.[17] In the absence of dexamethasone, we have seen a statistically significant ($P < .025$) increase in leptin release due to 1 μmol/L $dPGJ_2$ (Fig 3). The increase (40%) was comparable to that seen with 1 μmol/L troglitazone (Fig 1). The presence of dexamethasone did not appear to affect the increase in leptin release due to 1 μmol/L $dPGJ_2$. However, at a concentration of 10 μmol/L, $dPGJ_2$ markedly inhibited leptin release in the presence of dexamethasone, which may be secondary to the pronounced decrease in adipose tissue metabolism, as reflected in the marked reduction in glucose conversion to lactate, as well as lipolysis (Fig 3).

Table 1. Stimulation of Leptin Release by Troglitazone in the Presence of Forskilin Plus Insulin Is Converted to an Inhibition in the Presence of Dexamethasone

	% Change From Basal		
	Without Troglitazone	With Troglitazone (10 μmol/L)	*P* Value* (of the difference)
Leptin release			
Without dexamethasone			
Forskolin (2 μmol/L)	−13.0 ± 6.3	+0.9 ± 12.5	.215
Forskolin (2 μmol/L) and insulin (0.1 nmol/L)	+0.1 ± 7.1	+25.1 ± 14.1	.049
With dexamethasone (200 nmol/L)			
Forskolin (2 μmol/L)	−32.9 ± 8.9	−44.5 ± 7.6	.257
Forskolin (2 μmol/L) and insulin (0.1 nmol/L)	−2.5 ± 9.5	−24.3 ± 9.0	.029
Lipolysis			
Without dexamethasone			
Forskolin (2 μmol/L)	+49.2 ± 11.1	+47.3 ± 10.3	.402
Forskolin (2 μmol/L) and insulin (0.1 nmol/L)	+31.6 ± 7.1	+33.8 ± 7.7	.530
With dexamethasone (200 nmol/L)			
Forskolin (2 μmol/L)	+65.8 ± 17.6	+48.9 ± 13.3	.075
Forskolin (2 μmol/L) and insulin (0.1 nmol/L)	+33.6 ± 12.1	+28.7 ± 12.0	.168

NOTE. Fragments of human subcutaneous adipose tissue (390 mg) were incubated for 48 hours in 5 mL of medium in the absence or presence of 200 nmol/L dexamethasone and insulin (0.1 nmol/L), forskolin (2 μmol/L), or troglitazone (10 μmol/L) as indicated. The values are from 14 paired experimental replications using fat from 14 different individuals. The percent changes due to added agents are the mean ± SEM of the paired differences. The basal value for leptin release was 121 ± 20 (mean ± SEM) ng/g of fat in the absence and 350 ± 60 ng/g in the presence of dexamethasone. The basal value for lipolysis based on glycerol release was 6.6 ± 0.4 μmol/g of fat in the absence and 6.2 ± 0.6 μmol/g in the presence of dexamethasone.

*Statistical significance of the difference due to troglitazone estimated using repeated measures analysis of variance.

The response of human adipose tissue to hormones or added agents is quite variable, and this is illustrated in the data shown in Fig 4. The increase in leptin release due to 3.3 μmol/L troglitazone in the absence of dexamethasone from 22 individuals is plotted in Fig 4 against the BMI. Six individuals showed a response to troglitazone in excess of 60% with respect to

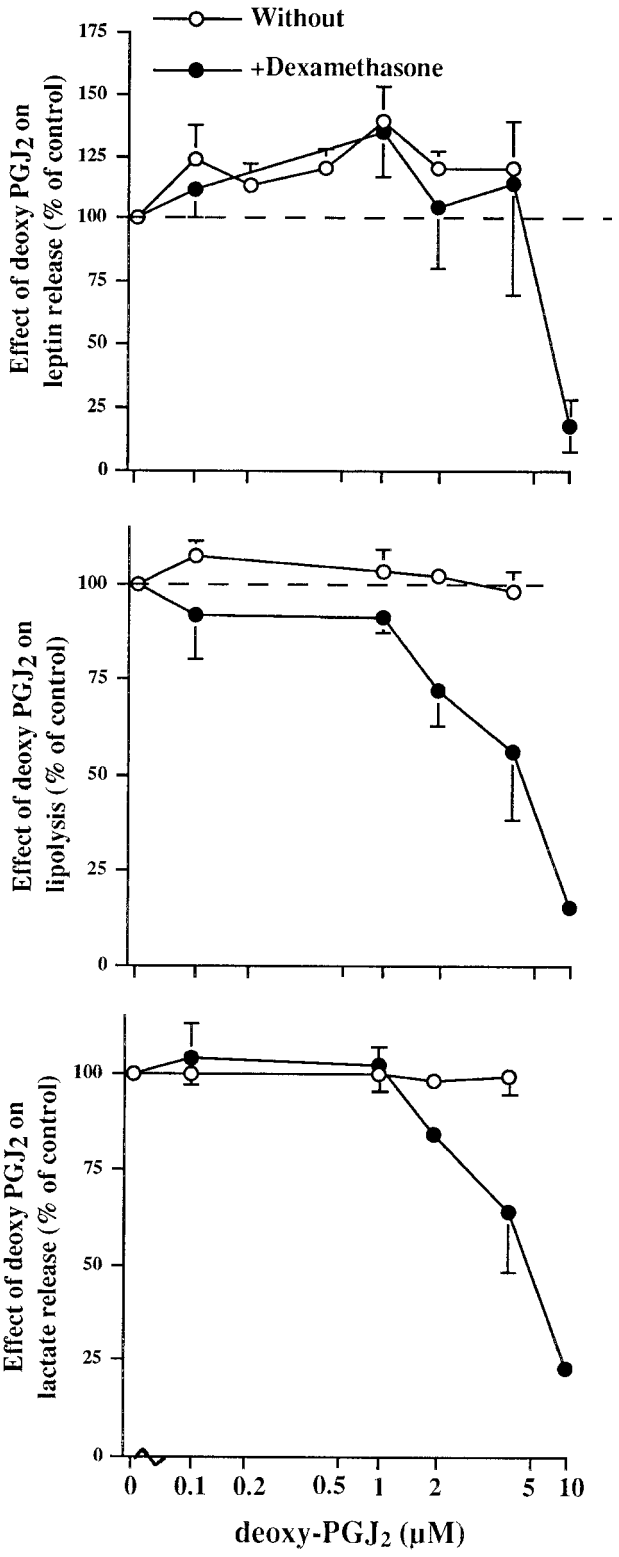

Fig 3. Stimulation of leptin release by low concentrations of deoxy PGJ_2. Fragments of human subcutaneous adipose tissue (~410 mg) were incubated for 48 hours in 5 mL of medium either without (○) or with 200 nmol/L dexamethasone (●). The values are shown as the percentage ± SEM of the control values for the effects of the indicated concentrations of $dPGJ_2$ and are for tissue from 6 different individuals.

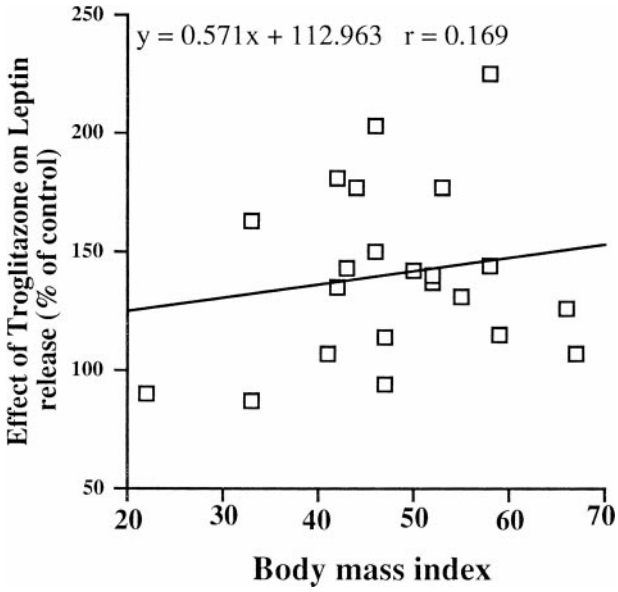

Fig 4. Lack of correlation between BMI and the effect of troglitazone on leptin release. Fragments of human adipose tissue (~395 mg) from 22 individuals were incubated for 48 hours in 5 mL of medium in the absence of dexamethasone either in the absence or presence of 3.3 μmol/L troglitazone. The effect of troglitazone on leptin release as percentage of the control value for leptin release by each individual is plotted *v* the BMI for that person.

stimulation of leptin release, while 13 had an intermediate response, and 3 had no response to troglitazone. Two of the adipose tissue samples that showed no response to troglitazone were from individuals with BMI values of 22 and 33 (formerly morbidly obese individuals who had gastric bypass operations a year previously and whose subcutaneous adipose tissue was obtained during abdominoplasty). There was no significant correlation between BMI and the response to troglitazone in adipose tissue from obese individuals (Fig 4). There was also no significant correlation between BMI and basal leptin release by adipose tissue from the individuals shown in Fig 4 (data not shown).

There was a significant correlation (0.62, $P < .005$) between the stimulation of leptin release by troglitazone in the absence of dexamethasone and that due to insulin (Fig 5). The data show that there was as much individual variation in the response to the insulin as to troglitazone.

DISCUSSION

The release of leptin to the medium over a 48-hour incubation of human adipose tissue represented new synthesis of leptin.[11] In this system, insulin stimulated leptin release, and the effect of 0.1 nmol/L insulin was 60% of that seen with 10 nmol/L insulin.[11] Similarly, an increase in leptin release from cut pieces of human subcutaneous fat by 7 nmol/L insulin was reported by Russell et al.[20] In both of our studies and those of Russell et al,[20] adipose tissue was incubated in serum-free media. In contrast, inhibitory effects of insulin on leptin release by cut pieces of human subcutaneous adipose tissue or adipocytes that were incubated for 48 hours have been reported in the presence of 10% sera.[21,22]

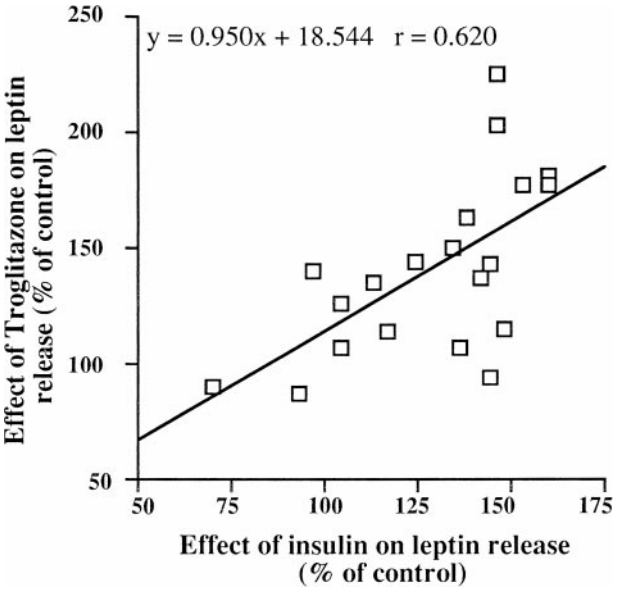

Fig 5. Correlation between the effect of troglitazone on leptin release and that of insulin. Fragments of adipose tissue (395 mg) were incubated for 48 hours in 5 mL of medium without dexamethasone either with 0.1 nmol/L insulin or 3.3 μmol/L troglitazone. The values are from 20 of the 22 individuals whose BMI is shown in Fig 4 and in which adipose tissue was incubated with insulin. The correlation coefficient was .62, which was significant with a $P < .005$.

Our data show a stimulation of leptin release by 3.3 μmol/L troglitazone in the absence of dexamethasone over a 48-hour incubation of human adipose tissue in primary culture. The stimulatory effect of troglitazone was dose-dependent because the effect of either 1 or 10 μmol/L troglitazone was about a half to two thirds that of 3.3 μmol/L troglitazone. No stimulatory effect of troglitazone was seen in the presence of dexamethasone, which itself increased release of leptin by 180%. Moreover, 10 μmol/L troglitazone actually blocked the stimulation of leptin release by insulin in the presence of dexamethasone (Fig 2), while inhibiting leptin release in the presence of dexamethasone, insulin, and forskolin (Table 1 and Fig 2). These data clearly illustrate the complex interactions of hormones and troglitazone with respect to leptin release.

Prior investigators using relatively high concentrations of thiazolidinediones (10 to 100 μmol/L) reported only inhibitory effects on leptin release. In the study of Nolan et al,[9] human adipocytes from 2 subjects were incubated for 72 hours in medium containing 10% fetal calf serum. Troglitazone at a concentration of 10 μmol/L was reported to markedly inhibit basal leptin release and to completely abolish the marked increase in leptin release due to insulin between 48 and 72 hours.[9] However, adipocytes in primary culture undergo a 40% loss of their 18S RNA over 24 hours.[12] This loss is much less if pieces of adipose tissue are incubated under the same conditions.[13]

The data in Fig 3 show that 1 μmol/L $dPGJ_2$ stimulated leptin release by cut pieces of human adipose tissue. $dPGJ_2$ is derived from PGJ_2, which in turn, is a product of PGD_2 metabolism. The common precursor of both prostaglandin E_2 (PGE_2) and PGD_2 is PGH_2, which is formed from arachidonic acid by cyclooxygenase enzymes. It has been reported that $dPGJ_2$ activates PPARγ, which is a *trans*-acting nuclear factor involved in adipocyte differentiation.[23,24] The effects of troglitazone and other thiazolidinediones have been attributed to their activation of PPARγ.[7] The amount of PPARγ-2 mRNAs is at least 10-fold higher in human subcutaneous adipose tissue than in rat or mouse adipose tissue.[25] There is also a positive correlation in humans between BMI and the amount of PPARγ-1 or -2 mRNAs in adipose tissue.[25] Small increases were seen in the amount of PPARγ-1 and -2 mRNA after a 48-hour incubation of human adipocytes with insulin plus dexamethasone.[25] Interestingly, if we incubated cut pieces of human adipose tissue for 48 hours with both insulin and dexamethasone, the addition of troglitazone abolished the effect of insulin on leptin release (Fig 2).

Our findings that both troglitazone and $dPGJ_2$ under appropriate conditions can directly stimulate leptin release by human adipose tissue are in contrast to those seen in cultured murine 3T3L1 cells in which the low release of leptin was inhibited by thiazolidinediones and $dPGJ_2$.[16,17] In adipose tissue from obese mice incubated for 24 hours in the same buffer used for human adipose tissue, we saw a 30% ± 9% ($n = 6$ experiments) increase in leptin release by 0.1 μmol/L $dPGJ_2$ (unpublished data). However, the effects of $dPGJ_2$ are very dose-dependent as shown in Fig 3 because $dPGJ_2$ at a concentration of 10 μmol/L actually inhibited leptin release in the presence of dexamethasone by human adipose tissue incubated for 48 hours.

It has recently been reported that $dPGJ_2$ and thiazolidinediones inhibited mitochondrial viability and induced apoptosis in cultured JEG_3 choriocarcinoma cells.[26] The dose-response relationship for inhibition of mitochondrial viability[26] was almost identical to that for inhibition of lipolysis and lactate release by human adipose tissue (Fig 3). These data suggest that the inhibitory effects of 10 μmol/L $dPGJ_2$ on leptin release may be secondary to induction of apoptosis. It is also possible that IKB kinase inhibition by 10 μmol/L $dPGJ_2$ could be involved in its toxic effects on adipose tissue.[13,19]

One long-term effect of treatment of obese Zucker rats with troglitazone is an increase in adipose tissue mass especially of small fat cells.[3] The increase in fat was accompanied by a marked decrease in the number of large fat cells and of leptin mRNA.[3] The increased proliferation of adipocytes is the expected long-term effect of activators of PPARγ, which was originally known as the adipocyte differentiation factor. It is unclear how leptin release is enhanced in human adipose tissue by low concentrations of troglitazone or $dPGJ_2$. However, as shown in Fig 3, this was not accompanied by any inhibition of lipolysis or glucose conversion to lactate. We saw similar findings with troglitazone in the experiments shown in Fig 1 in which lactate formation averaged 54 μmol/g over 48 hours in the absence of dexamethasone, but 58 μmol/g in the presence of 3.3 or 10 μmol/L troglitazone (unpublished data).

In conclusion, our data show that in pieces of human subcutaneous adipose tissue from morbidly obese individuals incubated for 48 hours in serum-free media, there was a stimulation of leptin release by troglitazone in the absence of

insulin or dexamethasone. Whether these findings are equally applicable to tissue from individuals with a normal BMI remains to be demonstrated. However, the stimulatory effect of troglitazone was not seen if dexamethasone or insulin were also present. Furthermore, an inhibitory effect of troglitazone on leptin release was seen in the presence of both insulin and dexamethasone.

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