An Osteoblast-dependent Mechanism Contributes to the Leptin Regulation of Insulin Secretion

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Our work focuses on genetic and molecular mechanisms for the reciprocal regulation of bone and energy metabolism orchestrated by leptin and osteocalcin. In the context of this reciprocal regulation, the finding that leptin inhibits insulin secretion by β cells while osteocalcin favors it is surprising. In exploring the molecular bases of this paradox we found that leptin, as is the case for most of its functions, uses a neuronal relay to inhibit insulin secretion. Cell-specific gene-deletion experiments revealed that a component of this neuronal regulation is the sympathetic innervation to osteoblasts. Under the control of leptin the sympathetic tone favors expression in osteoblasts of Esp, which inhibits the metabolic activity of osteocalcin. We further identify ATF4 as a transcription factor that regulates Esp expression and thereby insulin secretion and sensitivity. Taken together these data illustrate the tight connections between bone remodeling and energy metabolism and add further credence to the notion that the osteoblast is a bona fide endocrine cell type.

Key words: **leptin; osteoblast; osteocalcin**

Leptin inhibits insulin secretion in part through a well-established direct effect on β-cells[1,2] and in part through indirect mechanisms that have not been fully elucidated.[1–4] Since hyperinsulinemia is observed in both leptin-deficient (*ob/ob*) and lipodystrophic (adipocyte-deficient) states,[5–7] we used *ob/ob*, adipocyte-deficient, and other mouse models with perturbed leptin signaling to determine which indirect mechanisms contribute to leptin regulation of insulin secretion.

Ob/ob mice were analyzed at birth and 1 and 2 weeks of age because at those ages body weight, fat mass, triglyceride level, and insulin sensitivity are not noticeably altered by the absence of leptin (Fig. 1A and B and Fig. S1). Thus, young *ob/ob* mice constitute a good genetic

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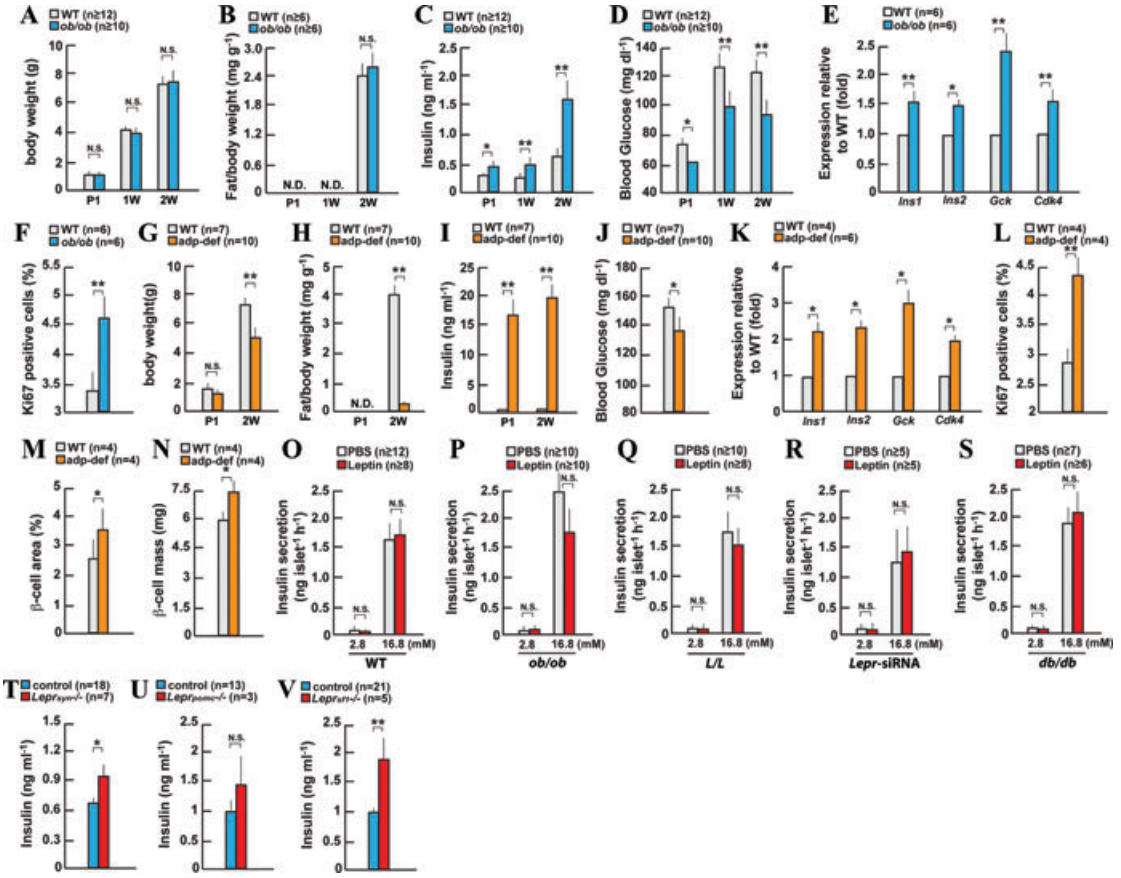

Figure 1. Leptin regulates, in part, insulin secretion through hypothalamic neuronal pathway. *Insulin* expression and secretion and β-cell proliferation in *ob/ob* (**A** to **F**) and adipocyte-deficient (adp-def) mice at indicated ages (**G** to **N**). (**A** and **G**) Body weight. (**B** and **H**) Fat mass. (**C** and **I**) Serum insulin levels. (**D** and **J**) Blood glucose levels. (**E** and **K**) Gene expression in pancreas or islets. (**F** and **L** to **N**) Insulin/Ki67-immunoreactive cells in islets and β-cell mass. (**O** to **S**) Glucose-stimulated insulin secretion in WT, *ob/ob*, *L/L*, *Lepr*-siRNA transfected WT, and *db/db* islets treated with leptin *in vitro*. (**T** to **V**) Serum insulin levels in $Lepr_{syn}^{-/-}$, $Lepr_{pomc}^{-/-}$ and $Lepr_{sf1}^{-/-}$ mice. Error bars, mean ± SEM. **, $P < 0.01$; *, $P < 0.05$; P1, newborn; 1W, 1 week-old; 2W, 2 week-old; N.S., not significant; N.D, not detected.

model to study, in the absence of metabolic abnormalities secondary to adiposity, mechanisms whereby leptin regulates insulin secretion. In 2-week-old *ob/ob* mice, serum insulin levels were 2.5-fold higher than in wild-type (WT) littermates, resulting in a more than 30% decrease of blood glucose levels after feeding (Fig. 1C and D). Remarkably, hyperinsulinemia and lower blood glucose levels were also present in newborn and 1-week-old *ob/ob* mice (Fig. 1C, D). These results established that leptin is a physiological regulator of serum insulin levels.

To understand how this severe hyperinsulinemia developed in mice otherwise metabolically normal, we studied islet gene expression and β-cell proliferation in 2-week-old WT and *ob/ob* mice. While insulin sensitivity appeared unaffected (Fig. S1B and C), expression of the *Insulin* genes and of *Glucokinase*,[8] a central component of the glucose-sensing machinery of β-cells, was increased 50% and 140%, respectively, in *ob/ob* mice (Fig. 1E), accounting, at least in part, for the increased insulin levels described above. Serum c-peptide levels were increased 2.5 fold in *ob/ob* mice (Fig. S2A).

Insulin content was also increased, albeit not significantly, in *ob/ob* pancreas (Fig. S3A). In addition, expression of *Cdk4,* a gene favoring β-cell proliferation *in vivo*,[9] was up-regulated 50% in *ob/ob* islets, and Ki67 immunostaining showed a significant increase in β-cell proliferation in *ob/ob* compared with WT mice (Fig. 1E and F). However, we could not detect an increase in β-cell area and β-cell mass (Fig. S4). This lack of increase in β-cell mass indicates that the absence of leptin affects circulating insulin levels primarily by regulating insulin expression and secretion in young *ob/ob* mice.

We asked if similar abnormalities were present in young lipodystrophic mice (adipocyte-deficient mice). Two-week-old adipocyte-deficient mice were markedly hyperinsulinemic and had a significant drop in blood glucose levels; this hyperinsulinemia was also observed at birth (Fig. 1G–J and Fig. S2B). Expression of *Insulin*, *Glucokinase,* and *Cdk4* was up-regulated to the same extent in islets of adipocyte-deficient and *ob/ob* mice (Fig. 1K). Moreover, insulin content was significantly increased in islets of adipocyte-deficient mice (Fig. S3B). In addition, β-cell proliferation measured by Ki67 immunostaining, β-cell area, and β-cell mass was significantly increased in lipodystrophic mice (Fig. 1L–N and Fig. S5). Taken together, these data show that hyperinsulinemia and lower blood glucose levels precede the appearance of obesity and hyperglycemia in *ob/ob* and adipocyte-deficient mice, respectively, and can be ascribed, in both cases, primarily to an increase in *Insulin* and *Glucokinase* expression and, to a lesser extent, to an increase in β-cell proliferation. These results are consistent with the notion that leptin inhibits *in vivo* insulin secretion. That hyperinsulinemia is far more severe in adipocyte-deficient than in *ob/ob* mice indicates that other mechanisms in addition to leptin deficiency affect insulin secretion in the former model.

Next we tested the influence of leptin on insulin secretion in isolated islets. Whether WT islets were cultured in low or high glucose concentration, we did not observe a decrease in the amount of insulin secreted in the medium following leptin treatment (Fig. 1O). When using *ob/ob* islets leptin did decrease insulin secretion (Fig. 1P), a result supporting the accepted notion that it acts directly on β-cells to regulate insulin secretion.[1,2] However, this decrease never reached statistical significance ($n \geq 10$). Thus, in the face of these results we asked whether islets isolated from mice harboring an activating mutation in the leptin receptor (*L/L* mice)[10] might be a better model to uncover a direct effect of leptin on β-cells. In that case also we failed to observe a significant decrease in insulin secretion following leptin treatment (Fig. 1Q). That insulin secretion was enhanced by high glucose concentration indicates that the lack of a statistically significant effect of leptin on insulin secretion in these experiments was not due to poor conditions of the islets. We also asked whether leptin would affect glucose-stimulated insulin secretion in islets that would not express its receptor. We first used siRNA to decrease *Lepr* expression, but failed to observe a change in glucose-stimulated insulin secretion after leptin treatment (Fig. 1R). Because siRNA decreased *Lepr* expression only 50% (data not shown), we were concerned that we did not create the right conditions to perform this experiment. Thus we also used islets isolated from *db/db* mice that harbor an inactivating mutation in the leptin receptor.[11] As shown in Fig. 1S leptin did not affect glucose-stimulated insulin secretion by *db/db* islets either. Taken together, data obtained through the use of islets isolated from WT and *ob/ob* mice and from gain- and loss-of-function mutations in the leptin receptor are all consistent with the hypothesis that the direct effect of leptin on β-cell may not be the only mechanism whereby it regulates insulin secretion.

In view of these results we asked whether leptin uses a neuronal relay, in addition to its direct effect on islets, to inhibit insulin secretion. To that end we made use of a floxed allele of the leptin receptor gene *Lepr* (*Lepr-flox/flox*).[12] Deletion of the leptin receptor in all neurons

through the use of *Synapsin-Cre* transgenic mice resulted in a mouse model harboring a significant increase in serum insulin levels (Fig. 1T). This result established that there was indeed a neuronal contribution to the leptin regulation of insulin secretion. In light of this observation we studied the involvement of the expression of the leptin receptor in two neuronal populations of the hypothalamus to the leptin regulation of insulin secretion. Deletion of the leptin receptor in arcuate neurons through the use of *Pomc-Cre* transgenic mice did not affect serum insulin levels (Fig. 1U). In contrast, deleting it from ventromedial hypothalamic (VMH) neurons through the use of *Sf1-Cre* transgenic mice resulted in a nearly 2-fold increase in serum insulin level in absence of insulin resistance (Fig. 1V and Fig. S6). These results indicate that VMH neurons contribute to the neuronal pathway whereby leptin regulates insulin secretion.

Since VMH neurons also contribute to leptin regulation of sympathetic tone,[13,14] we then asked whether sympathetic tone could be a mediator of leptin-dependent central regulation of insulin secretion.[4,15] First we analyzed expression of all adrenergic receptors in islets. *Adr*β*1* and *Adr*β*2* were by far the most highly expressed; *Adr*α*2A* was also expressed, but at a 5-fold lower level. None of the other adrenergic receptors were detected in islets (Fig. S7). Second, we treated WT, *ob/ob*, and adipocyte-deficient newborn pups for 2 weeks with either isoproterenol, a sympathomimetic acting through β-adrenergic receptors; clonidine, a sympathomimetic acting through the Adrα2 receptor; or, as a negative control, phenylephrine, a sympathomimetic acting through the Adrα1 receptor. As expected phenylephrine did not affect serum insulin levels in any of the mouse models tested, and clonidine had only a marginal inhibitory effect (Fig. 2A). In contrast, isoproterenol decreased hyperinsulinemia by more than half in *ob/ob* and adipocyte-deficient but not in WT mice (Fig. 2A and B). That *Ucp1* expression was increased by isoproterenol supports the notion that there was an increase in sympathetic tone as a result of this treatment (Fig. S8). Remarkably, however, isoproterenol, like phenylephrine and clonidine did not influence glucose-stimulated insulin secretion from isolated islets maintained in either low or high concentration of glucose (Fig. 2C). Isoproterenol did not affect gene expression in WT islets either (Fig. S9). Isoproterenol treatment of *ob/ob* or *L/L* islets also failed to affect glucose-stimulated insulin secretion (Fig. 2D and E). Collectively, these observations indicate that sympathetic tone acting through β-adrenergic receptors regulates insulin secretion but are inconsistent with a model whereby it would do so by acting directly on islets.

The lack of a noticeable effect of isoproterenol on glucose-stimulated insulin secretion in islets and the link between VMH neurons, sympathetic tone, and bone mass, together with the fact that *Adr*β*2* is the only adrenergic receptor expressed in osteoblasts,[13] were three facts that led us to test the hypothesis that it was through the osteoblasts that sympathetic tone, under the control of leptin, regulates insulin secretion. To test this hypothesis we analyzed various mutant mouse strains each lacking one adrenergic receptor. While serum insulin levels were normal in *Adr*β*1*$^{-/-}$ and *Adn*α*2A*$^{-/-}$ mice, they were significantly higher in mice lacking *Adr*β*2* in osteoblasts only (*Adr*β$2_{osb}{}^{-/-}$ mice) than in WT mice (Fig. 2F–H and Fig. S10). This abnormality developed in *Adr*β$2_{osb}{}^{-/-}$ mice in the absence of insulin resistance (Fig. S11). *Adr*β$2_{osb}{}^{-/-}$ mice had also a 80% to 150% increase in *Insulin* and *Glucokinase* expression and exhibited postprandial low blood glucose levels (Fig. 2I and J), all abnormalities observed in *ob/ob* mice. Likewise, and as seen in *ob/ob* and adipocyte-deficient mice, *Cdk4* expression was increased 50% in *Adr*β$2_{osb}{}^{-/-}$ mice (Fig. 2J and Fig. S12A).

To establish formally that the increase in insulin circulating levels noted in the *Adr*β$2_{osb}{}^{-/-}$ mice occurs under the control of leptin, we generated *ob/+*;*Adr*β$2_{osb}{}^{+/-}$ compound mutant mice. While both *Adr*β$2_{osb}{}^{+/-}$ and *ob/+* mice had normal serum insulin

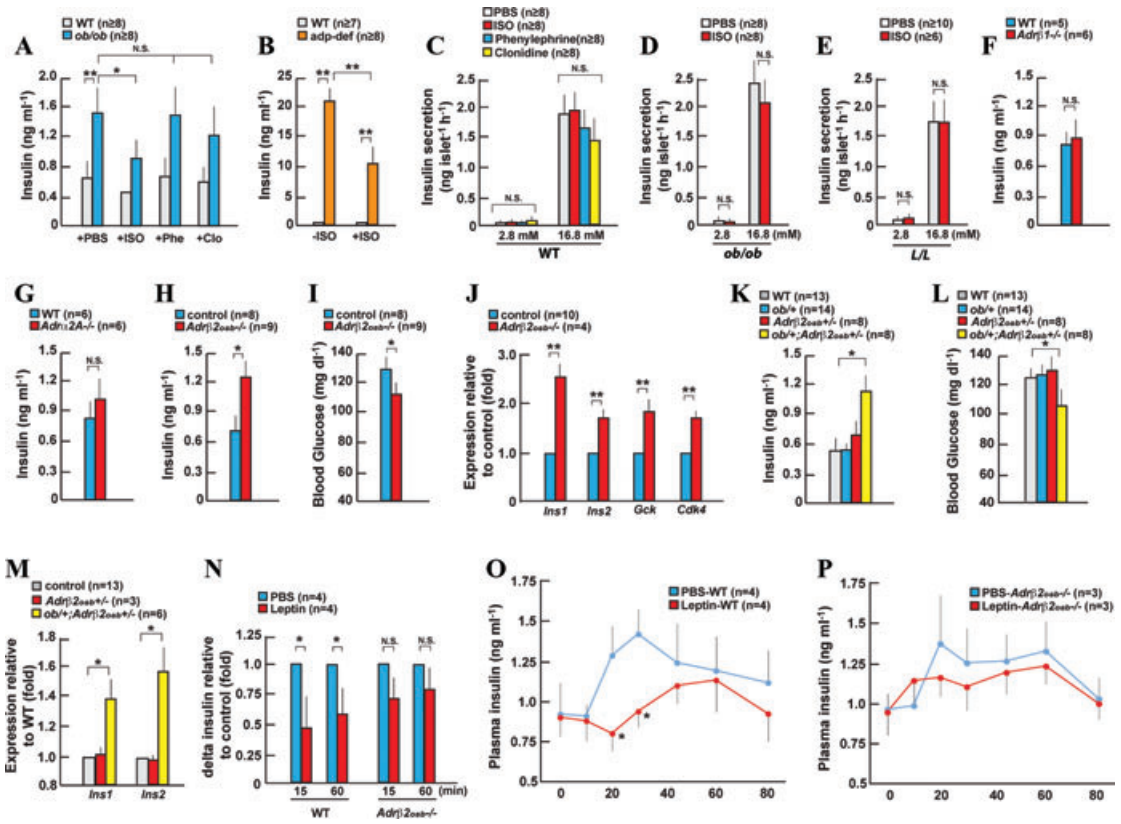

Figure 2. Sympathetic signaling in osteoblasts regulates insulin expression and secretion. (**A** and **B**) Serum insulin levels in *ob/ob* and adipocyte-deficient mice after daily isoproterenol, phenylephrine, or clonidine injection for 2 weeks. (**C** to **E**) Glucose-stimulated insulin secretion in WT, *ob/ob*, and *L/L* islets treated with isoproterenol, phenylephrine, or clonidine *in vitro*. (**F** and **G**) Serum insulin levels in $Adr\beta1^{-/-}$ or $Adr\alpha2A^{-/-}$ mice. (**H** to **J**) Serum insulin and blood glucose levels and gene expression in islets of 2-week-old $Adr\beta2_{osb}{}^{-/-}$ mice. (**K** to **M**) Serum insulin, blood glucose, and *Insulin* expression in pancreas of 2-week-old *ob/+*;$Adr\beta2_{osb}{}^{+/-}$ mice. (**N**) Delta insulin after glucose-stimulated insulin secretion in WT and $Adr\beta2_{osb}{}^{-/-}$ mice treated with central leptin infusion at 4 ng/h for 1 week. (**O** and **P**) Plasma insulin levels during hyperglycemic clamps in WT and $Adr\beta2_{osb}{}^{-/-}$ mice treated with central leptin infusion at 4 ng/h for 1 week. Error bars, mean ± SEM; **, $P < 0.01$; *, $P < 0.05$. Control in **H–J** and **M** indicates *α1(I)Collagen-Cre* mice.

levels, *ob/+*;$Adr\beta2_{osb}{}^{+/-}$ mice displayed hyperinsulinemia, lower blood glucose level after feeding, and increased *Insulin* expression (Fig. 2K–M and Fig. S12B), as did $Adr\beta2_{osb}{}^{-/-}$ and *ob/ob* mice. To further establish that sympathetic tone in osteoblasts contributes to the leptin regulation of insulin secretion, we performed two additional experiments. First, long-term (1 week) intracerebroventricular (ICV) infusion of leptin (4 ng/h) decreased glucose-stimulated insulin secretion in WT but not in $Adr\beta2_{osb}{}^{-/-}$ mice (Fig. 2N). This effect of leptin was not observed when it was delivered peripherally at that dose (data not shown). Additionally, we performed a hyperglycemic clamp to assess glucose-stimulated insulin secretion *in vivo*. Similar to the previous observation, leptin ICV infusion during hyperglycemic clamps decreased circulating insulin levels in WT but not $Adr\beta2_{osb}{}^{-/-}$ mice (Fig. 2O, P). Taken together, these observations identify sympathetic signaling in osteoblasts as a significant mediator of leptin's regulation of insulin secretion.

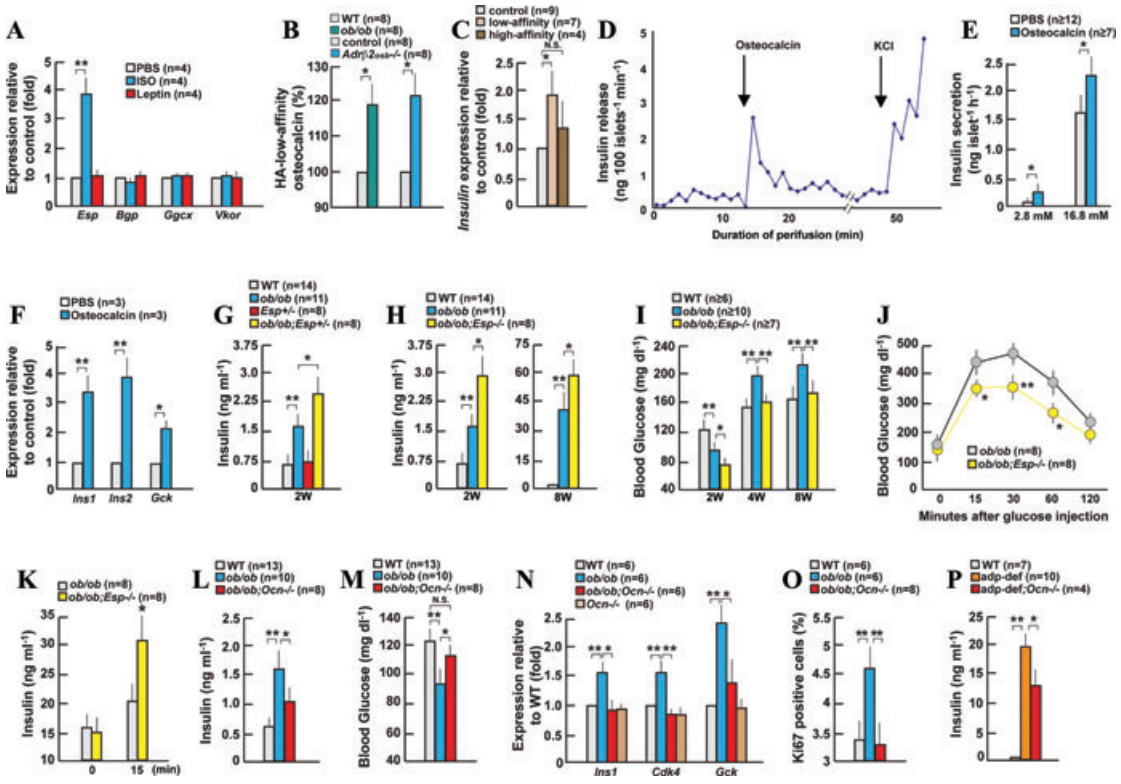

Figure 3. Leptin regulates insulin secretion through osteoblasts. (**A**) Gene expression in isoproterenol- or leptin-treated osteoblasts. (**B**) Levels of uncarboxylated osteocalcin (HA low affinity) in *ob/ob* and $Adr\beta2_{osb}^{-/-}$ mice. (**C**) *Insulin* expression by HA-affinity fraction in islets. (**D**) Perifusion of WT islets in the presence of osteocalcin (0.03 ng/mL) or KCl under 3 mM glucose condition. (**E**) Insulin secretion by osteocalcin in WT islets maintained in low and high glucose. (**F**) Gene expression in osteocalcin-treated islets. (**G** to **I**) Serum insulin and blood glucose in 2- to 8-week-old $ob/ob;Esp^{+/-}$ and $ob/ob;Esp^{-/-}$ mice. (**J**) GTT in 2-month-old $ob/ob;Esp^{-/-}$ mice. (**K**) Serum insulin after glucose challenge in 2-month-old $ob/ob;Esp^{-/-}$ mice. (**L** and **M**) Serum insulin and blood glucose in 2-week-old $ob/ob;Ocn^{-/-}$ mice. (**N** and **O**) Insulin/Ki67-positive cells and gene expression in pancreas or islets of $ob/ob;Ocn^{-/-}$ mice. (**P**) Serum insulin in 2-week-old adp-def;$Ocn^{-/-}$ mice. Error bars, mean ± SEM; **, $P < 0.01$; *, $P < 0.05$ Control in B indicates $\alpha 1(I)Collagen\text{-}Cre$ mice.

How does sympathetic signaling in osteoblasts regulate insulin secretion in β-cells? Osteoblasts secrete osteocalcin, a molecule stimulating β-cell proliferation and *Insulin* expression, whose biological activity is decreased by the product of the *Esp* gene.[16] Multiple observations indicate that the leptin-dependent sympathetic regulation of insulin secretion occurs by modulating osteocalcin bioactivity.

Esp expression was up-regulated 4-fold by isoproterenol in osteoblasts, while the expression of *Osteocalcin* and other enzymes modifying it was not affected (Fig. 3A). Accordingly, and although serum level of total osteocalcin was normal (Fig. S13), the amount of osteocalcin binding to hydroxyapatite (HA) with low affinity (that is, under-carboxylated and active biologically[16]) was increased in both *ob/ob* and $Adr\beta2_{osb}^{-/-}$ mice (Fig. 3B). Two control experiments validated this finding. First, when using serum containing only uncarboxylated osteocalcin as a negative control, osteocalcin eluted in the same fractions as the ones harboring an increased content of osteocalcin in *ob/ob* serum (data not shown). Second, the low-affinity fractions could regulate *Insulin* expression in isolated islets while the fractions binding to HA with high affinity could not (Fig. 3C).

TABLE 1. Insulin Sensitivity Analysis of 2-month-old *ob/ob* and *ob/ob;Esp*$^{-/-}$ Mice Analyzed by Hyperinsulinemic-Euglycemic Clamps

	Wild-type ($n = 4$)	*ob/ob* ($n = 4$)	*ob/ob;Esp*$^{-/-}$ ($n = 4$)
Body weight (g)	17.8 ± 0.4	38.0 ± 1.7	31.5 ± 1.0
Glucose infusion rate (mg/kg/min)	64.9 ± 4.7	0.0 ± 0.0	0.0 ± 0.0
Basal hepatic glucose production (mg/kg/min)	15.9 ± 2.9	17.0 ± 1.2	15.1 ± 3.1
Clamp hepatic glucose production (mg/kg/min)	−1.0 ± 4.2	20.7 ± 0.4	23.0 ± 1.6
Glucose turnover (mg/kg/min)	63.9 ± 2.5	20.7 ± 0.4	23.0 ± 1.6
Glycogen synthesis (mg/kg/min)	30.3 ± 1.9	4.4 ± 2.6	1.1 ± 0.4
Muscle glucose uptake (mL/kg/min)	65.4 ± 3.3	25.2 ± 5.7	22.3 ± 1.9
White adipose glucose uptake (mL/kg/min)	4.5 ± 0.9	1.2 ± 0.3	1.4 ± 0.2

Next we tested whether osteocalcin regulates insulin secretion directly. In an islets perifusion assay, osteocalcin (0.03 ng/mL) was a powerful insulin secretagogue (Fig. 3D) with a strong first phase and sustained, but lower, second phase of insulin secretion in all experiments performed. Osteocalcin also augmented glucose-stimulated insulin secretion from isolated islets maintained in either low or high glucose concentrations (Fig. 3E). Moreover, and unlike isoproterenol, osteocalcin treatment of isolated islets increased expression of *Insulin* and *Glucokinase* (Fig. 3F). *Insulin* expression was induced by osteocalcin in a concentration-dependent manner,[17] the effect was maximal at 4 h and disappeared at 8 h (Fig. S14).

If osteocalcin is a mediator of the leptin-dependent sympathetic regulation of insulin secretion then increasing its bioactivity should worsen hyperinsulinemia in *ob/ob* mice. To test this contention we generated *ob/ob* mice lacking one allele of *Esp*, the gene inhibiting osteocalcin bioactivity (*ob/ob;Esp*$^{+/-}$ mice) and noticed in these mice a significant increase in serum insulin levels when compared with *ob/ob* mice (Fig. 3G). Since *Esp*$^{+/-}$ mice have normal circulating insulin levels, this result established the existence of a genetic interaction between *Leptin* and *Esp*. We also generated *ob/ob* mice lacking both alleles of *Esp* (*ob/ob;Esp*$^{-/-}$ mice). Most of these mutant mice died with severe hypoglycemia within the first few days of life (Fig. S15). The surviving *ob/ob;Esp*$^{-/-}$ mice were two times more hyperinsulinemic than *ob/ob* mice at 2 weeks of age. They also had low blood glucose levels and remained significantly less glucose intolerant than *ob/ob* mice until 8 weeks of age, illustrating the importance of this pathway in adult animals (Fig. 3H–J and Fig. S16). Improved glucose tolerance was demonstrated by the ability of a glucose challenge to increase insulin secretion in *ob/ob;Esp*$^{-/-}$ but not in *ob/ob* mice (Fig. 3K), while insulin sensitivity, as measured by hyperinsulinemic-euglycemic clamps, was similar in *ob/ob;Esp*$^{-/-}$ and *ob/ob* mice (Table 1).

Lastly we tested the role of osteocalcin as a mediator of leptin regulation of insulin secretion. Mice lacking both *Leptin* and *Osteocalcin* (*ob/ob*;*Osteocalcin*$^{-/-}$) had serum insulin levels 50% lower than *ob/ob* mice and were normoglycemic at 2 weeks of age (Fig. 3L and M). Expression of *Insulin*, *Glucokinase*, and *Cdk4* was decreased 50% to 100% in *ob/ob;Osteocalcin*$^{-/-}$ compared with *ob/ob* mice, and β-cell proliferation was normalized in *ob/ob;Osteocalcin*$^{-/-}$ mice (Fig. 3N and O). It is worth noting that the correction of hyperinsulinemia was similar in isoproterenol-treated *ob/ob* and *ob/ob*;*Osteocalcin*$^{-/-}$ mice, suggesting that osteocalcin is a mediator of the leptin-dependent sympathetic regulation of insulin secretion. In addition, osteocalcin deletion in adipocyte-deficient mice decreased their serum insulin levels by half (Fig. 3P). Taken together,

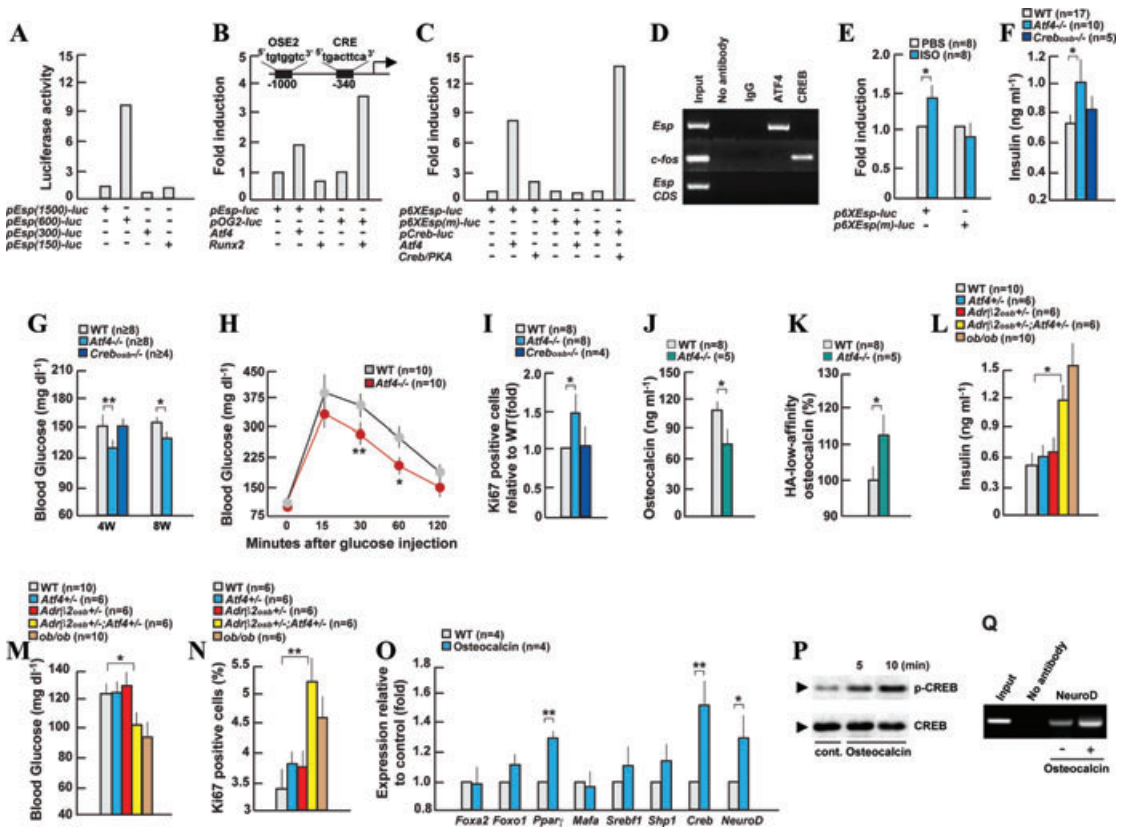

Figure 4. Sympathetic tone regulates osteocalcin bioactivity through *Atf4*. (**A**) *Esp* promoter activity in ROS17/2.8 osteoblasts. (**B** and **C**) Cotransfection assay in COS cells using *Esp* promoter and indicated expression vectors. (**D**) ChIP assay in osteoblasts. (**E**) *Esp* promoter activity in ROS17/2.8 osteoblasts treated with isoproterenol (10 μM). (**F** and **G**) Serum insulin and blood glucose levels in $Creb_{osb}^{-/-}$ and $Atf4^{-/-}$ mice at indicated ages. (**H**) GTT in 2-month-old $Atf4^{-/-}$ mice. (**I**) Insulin/Ki67-positive cells in islets of WT, $Creb_{osb}^{-/-}$, and $Atf4^{-/-}$ mice. (**J** and **K**) Total and uncarboxylated (HA low affinity) osteocalcin levels in $Atf4^{-/-}$ mice. (**L** to **N**) Serum insulin, blood glucose levels and insulin/Ki67-immunoreactive cells in islets of $Adr\beta2_{osb}^{+/-}$;$Atf4^{+/-}$ mice. (**O**) Gene expression in islets treated with osteocalcin for 2 h. (**P**) Phosphorylation of CREB in osteocalcin-treated β-cells. (**Q**) ChIP assay in β-cells. Error bars, mean ± SEM; **, $P < 0.01$; *, $P < 0.05$.

these results support the hypothesis that the leptin-dependent sympathetic regulation of insulin secretion occurs via the osteoblast and uses osteocalcin as its mediator. Although we do not know at present how *Esp* modulates osteocalcin carboxylation, the most likely explanation is that it affects the activity of gamma carboxylase, the enzyme necessary for carboxylation of osteocalcin, through an indirect mechanism since this enzyme is not phosphorylated (data not shown).

How does sympathetic tone regulate *Esp* expression and osteocalcin bioactivity? *Esp* contains a cAMP-responsive element (CRE) at −340 bp that is required, in cell-based assays, for osteoblast-specific and sympathetic-dependent activity of this promoter fragment (Fig. 4A–C and E). Chromatin immunoprecipitation assays demonstrated that this CRE is not occupied by CREB itself, but by ATF4, an osteoblast-specific member of the CREB family whose activity is regulated by PKA (Fig. 4D).[18] The functional relevance of ATF4 binding to the *Esp* promoter in regulating glucose metabolism was documented *in vivo*. $Atf4^{-/-}$ mice exhibited significant postprandial

hyperinsulinemia and low blood glucose level, along with an increase in glucose tolerance and β-cell proliferation (Fig. 4F–I). In contrast none of these abnormalities was observed in mice lacking CREB in osteoblasts only (Fig. 4F, G and I and Fig. S17). *Atf4*$^{-/-}$ mice also displayed a relative increase in the percentage of uncarboxylated (*i.e.*, bioactive) osteocalcin, despite the fact that total osteocalcin blood levels were lower in these mice, an expected finding since ATF4 regulates *Osteocalcin* expression (Fig. 4J, K).[18] To provide evidence that *Atf4* mediates the sympathetic regulation of insulin secretion through its expression in osteoblasts, we generated compound heterozygous mice lacking, in osteoblasts, one copy of *Adrβ2* and one copy of *Atf4*. These *Adrβ2*$_{osb}^{+/-}$;*Atf4*$^{+/-}$ mice showed a doubling in circulating insulin levels leading to hypoglycemia and an increase in β-cell proliferation, all features comparable to what was observed in *ob/ob* mice (Fig. 4L–N). To address the molecular mechanisms used by osteocalcin to regulate insulin expression and secretion, we studied expression of genes encoding transcription factors regulating *Insulin* and *Glucokinase*.[19] As shown here osteocalcin induced within 2 h expression of *Creb* and *NeuroD,* two well-known regulators of *Insulin* expression[19] (Fig. 4O). Osteocalcin treatment also increased phosphorylation of CREB, an event necessary for its transcriptional activity, and binding of NeuroD to its cognate sequence on the insulin promoter (Fig. 4P and Q).[20] Besides *NeuroD* and *Creb*, osteocalcin treatment of β-cells also enhanced expression of *Pparγ* (Fig. 4O), a known regulator of glucokinase expression.[21]

Taken together the *in vivo* data presented here (Table S1) indicate that for approximately half of it leptin's inhibition of insulin secretion relies on a 3-step cascade including: i) leptin's upregulation of sympathetic tone, ii) sympathetic enhancement of *Esp* expression in osteoblasts, and iii) decrease in osteocalcin bioactivity. Our results do not deny that leptin can act directly on β-cells to regulate insulin secretion; instead they point toward an additional mechanism accounting for leptin regulation of insulin secretion *in vivo*. This mechanism of action relies, as it is the case for other functions of leptin,[22,23] on a central relay, but also includes another target organ of leptin, namely, bone. The absence of increase in β-cell mass in young *ob/ob* mice suggests that osteocalcin acts in this context primarily by regulating insulin expression and secretion. Beyond the leptin/sympathetic, tone/osteocalcin/insulin cascade, these results establish that the metabolic connection between adipocytes and osteoblasts recently uncovered is not limited to cross-regulation.[16,24,25] Rather, this cross-talk includes, as an important component, the use by adipocytes of osteoblasts to mediate a significant part of one of their endocrine functions on β-cells. This unexpected functional relationship between adipocytes, sympathetic tone, and osteoblasts illustrates *in vivo* the importance of the skeleton in regulating insulin secretion and glucose homeostasis.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Absence of metabolic disturbance in 2 week-old *ob/ob* mice.

Figure S2. Serum c-peptide in 2 week-old *ob/ob* and adipocyte-deficient mice.

Figure S3. Insulin content in pancreas of 2 week-old *ob/ob* and adipocyte-deficient mice.

Figure S4. β-cell area and β-cell mass in 2 week-old *ob/ob* mice.

Figure S5. Insulin immunostaining in pancreas of 2 week-old adipocyte-deficient mice.

Figure S6. ITT in 1 month-old $Lepr_{sf1}$-/- mice.

Figure S7. Adrenergic receptor expression in islets and osteoblasts.

Figure S8. *Ucp1* expression in brawn fat of *ob/ob* and adipocyte-deficient mice after daily isoproterenol injection for 2 weeks.

Figure S9. Gene expression by isoproterenol in islets.

Figure S10. Generation of $Adr\beta2_{osb}$-/- mice.

Figure S11. ITT in 2 month-old $Adr\beta2_{osb}$-/- mice.

Figure S12. Insulin/Ki67-immunoreactive cells in islets of 2 week-old (A) $Adr\beta2_{osb}$-/- and (B) $ob/+;Adr\beta2_{osb}+/-$ mice.

Figure S13. Total serum osteocalcin in 2 week-old *ob/ob* and $Adr\beta2_{osb}$-/- mice.

Figure S14. Time course of *Insulin* expression by uncarboxylated osteocalcin in islets.

Figure S15. Lethality of $ob/ob;Esp^{-/-}$ pups.

Figure S16. Serum c-peptide in $ob/ob;Esp^{-/-}$ mice.

Figure S17. GTT in 3 month-old $Creb_{osb}$-/- mice.

Table S1. Mutant mice used in this study and their phenotypes.

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Conflicts of Interest

The authors declare no conflicts of interest.

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