The sympathetic tone mediates leptin's inhibition of insulin secretion by modulating osteocalcin bioactivity

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The osteoblast-secreted molecule osteocalcin favors insulin secretion, but how this function is regulated in vivo by extracellular signals is for now unknown. In this study, we show that leptin, which instead inhibits insulin secretion, partly uses the sympathetic nervous system to fulfill this function. Remarkably, for our purpose, an osteoblast-specific ablation of sympathetic signaling results in a leptin-dependent hyperinsulinemia. In osteoblasts, sympathetic tone stimulates expression of *Esp*, a gene inhibiting the activity of osteocalcin, which is an insulin secretagogue. Accordingly, *Esp* inactivation doubles hyperinsulinemia and delays glucose intolerance in *ob/ob* mice, whereas *Osteocalcin* inactivation halves their hyperinsulinemia. By showing that leptin inhibits insulin secretion by decreasing osteocalcin bioactivity, this study illustrates the importance of the relationship existing between fat and skeleton for the regulation of glucose homeostasis.

Introduction

Our understanding of the mechanisms regulating insulin secretion at the organismal level improved greatly recently with the identification of several novel hormones (Herman and Kahn, 2006; Gesta et al., 2007; Lee et al., 2007). It was shown, for instance, that skeleton acts as an endocrine regulator of energy metabolism through the osteoblast-specific secreted molecule osteocalcin that favors insulin secretion by β cells, insulin sensitivity in fat, liver, and muscle, and energy expenditure (Lee et al., 2007). Although osteocalcin bioactivity is regulated by at least two gene products within the osteoblast, Esp and γ-carboxylase (Bügel, 2008), it remains unknown whether extracellular cues regulate its secretion or function.

In contrast to osteocalcin, leptin inhibits insulin secretion in part through a direct effect on β cells (Covey et al., 2006; Morioka et al., 2007) and, as is the case for most of its functions, in part through indirect mechanisms (Friedman and Halaas, 1998; Kieffer and Habener, 2000). Leptin affects osteoblast functions, raising the testable hypothesis that it could inhibit insulin secretion by decreasing osteocalcin activity (Ducy et al., 2000; Takeda et al., 2002).

In this study, we show that one important mechanism whereby leptin inhibits insulin secretion is by inhibiting the bioactivity of osteocalcin. These results provide in vivo evidence of the importance of the cross talk existing between osteoblasts and adipocytes in glucose homeostasis.

Results and discussion

Regulation of insulin secretion by leptin

The fact that leptin and osteocalcin exert opposite functions on insulin secretion prompted us to test whether they act independently of each other or not. To avoid the confounding issue of insulin resistance, we analyzed insulin secretion in leptin-deficient (*ob/ob*) mice at birth and 1 and 2 wk of age because at those ages body weight, abdominal and total fat mass, triglyceride level, and insulin sensitivity are not altered by the absence

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Abbreviations used in this paper: HA, hydroxyapatite; ICV, intracerebroventricular; ITT, insulin tolerance test; WT, wild type.

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of leptin (Fig. 1 A and Fig. S1, A–D, available at http://www.jcb.org/cgi/content/full/jcb.200809113/DC1).

In 2-wk-old *ob/ob* mice serum, insulin levels were 2.5-fold higher than in wild-type (WT) littermates, resulting in a >30% decrease of blood glucose levels after feeding. Remarkably, hyperinsulinemia and low blood glucose levels were also present in newborn and 1-wk-old *ob/ob* mice (Fig. 1, B and C). To understand how this marked hyperinsulinemia develops in mice that are otherwise metabolically normal, we studied islet gene expression and β-cell proliferation in WT and *ob/ob* mice. Expression of the *Insulin* genes and of *Glucokinase*, a central component of the glucose-sensing machinery of β cells (Grupe et al., 1995), was increased 50% and 140%, respectively, in *ob/ob* mice (Fig. 1 D), at least partly explaining the aforementioned increased insulin levels. Serum c-peptide levels were increased 2.5-fold in *ob/ob* mice (Fig. S1 E). There was also a small but detectable and reproducible increase in insulin content in *ob/ob* pancreata (Fig. S1 F). In addition, expression of *Cdk4*, a gene favoring β-cell proliferation in vivo (Rane et al., 1999), 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. 1, D and E). Undetectable increases in β-cell area and β-cell mass (Fig. S1, G and H) indicate that the absence of leptin affects circulating insulin levels primarily by regulating insulin expression and secretion in addition to the possibility of changes of cell survival. Nevertheless, these results establish that leptin is a physiological regulator of serum insulin levels independent of the influence it may have on insulin sensitivity.

We asked whether similar abnormalities were present in mice lacking adipocytes altogether. 2-wk-old adipocyte-deficient mice were also markedly hyperinsulinemic and had a significant drop in blood glucose levels; this hyperinsulinemia was also observed at birth (Fig. 1, F and G; and Fig. S1, I and J). Expression of *Insulin*, *Glucokinase*, and *Cdk4* was up-regulated to the same extent in islets of adipocyte-deficient and *ob/ob* mice (Fig. 1 H), and insulin content was significantly increased in islets of adipocyte-deficient mice (Fig. S1 K). β-cell proliferation, area, and mass were all significantly increased in adipocyte-deficient mice (Fig. 1, I–K; and Fig. S1 L). Collectively, and although insulin resistance may have favored β-cell proliferation in the adipocyte-deficient mice, the data presented here 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 to an increase in *Insulin* and *Glucokinase* expression and, to a lesser extent, to an increase in β-cell proliferation. More severe hyperinsulinemia in adipocyte-deficient mice than in ob/ob mice indicates that in addition to leptin deficiency, other mechanisms affect insulin secretion in the former model.

Multiple mechanisms contribute to leptin regulation of insulin secretion

Next, we studied leptin influence on isolated islets. When using WT islets cultured in low or high glucose concentration, we failed to observe a decrease in the amount of insulin secreted in the medium after leptin treatment. In contrast, leptin did decrease insulin secretion by *ob/ob* islets (Fig. 1, L and M). This result supports the notion that this hormone acts directly on β cells to regulate insulin secretion; however, the decrease observed was too modest to explain why the absence of leptin would result in a 2.5-fold increase in circulating insulin levels (Fig. 1 B). In the face of these results, we asked whether islets isolated from mice harboring an activating mutation in the leptin receptor (*L/L* mice; Björnholm et al., 2007) might be a better tool to uncover a more significant direct effect of leptin on β cells. In this study, again we observed only a modest decrease in insulin secretion after leptin treatment (Fig. 1 N). The marked increase in insulin secretion by high glucose concentration served as an internal positive control and indicated that the islets used in all of these experiments were in good condition. Collectively, data obtained using islets isolated from WT, *ob/ob*, and *L/L* mice verified that leptin acts locally on β cells to inhibit insulin secretion but were also consistent with the hypothesis that in addition to this direct effect, leptin may use indirect mechanisms to regulate insulin secretion.

Leptin-dependent sympathetic regulation of insulin secretion

Given what is known about the mediation of most of its functions, the aforementioned results prompted us to ask whether leptin also uses a neuronal relay to inhibit insulin secretion. Thus, we crossed mice harboring a floxed allele of the leptin receptor gene *Lepr* (*Lepr-flox/flox*) with *Synapsin-Cre* transgenic mice to remove the leptin receptor in all neurons (van de Wall et al., 2008). 1-mo-old *Synapsin-Cre*;*Lepr-flox/flox* ($Lepr_{syn}^{-/-}$) mice had a normal body weight but harbored a significant increase in serum insulin levels, indicating that in vivo there is a significant neuronal contribution to the leptin regulation of insulin secretion (Fig. 1 O and Fig. S1 M).

We noted that in $Lepr_{syn}^{-/-}$ mice, sympathetic tone, as measured by *Ucp1* expression in brown fat and serum levels of epinephrine and norepinephrine, was low (Fig. 2, A–C). This decrease, similar to the one observed in *ob/ob* mice, led us to ask whether the sympathetic tone could be a mediator of the leptin-dependent neuronal regulation of insulin secretion (Berthoud and Jeanrenaud, 1979). WT, *ob/ob*, and adipocyte-deficient newborn pups were treated for 2 wk with sympathomimetics acting through β-adrenergic receptors (isoproterenol) or through the Adrα2 (clonidine) or Adrα1 (phenylephrine) receptors. Neither phenylephrine nor clonidine affected serum insulin levels in any of the mouse models tested; in contrast, isoproterenol used at a moderate dose (3 mg/kg) significantly decreased circulating insulin levels in *ob/ob* and adipocyte-deficient mice (Fig. 2, D and E). Isoproterenol increased *Ucp1* expression in brown fat, supporting the notion that there was an increase in sympathetic tone following this treatment (Fig. 2 F). Isoproterenol did not affect circulating insulin in WT mice, probably because it was used at a dose that did not cause a massive increase in sympathetic tone. Indeed, *Ucp1* expression was not significantly increased in WT mice by isoproterenol (Fig. 2 F). Collectively, these observations establish that the sympathetic tone acting through β-adrenergic receptors regulates insulin secretion.

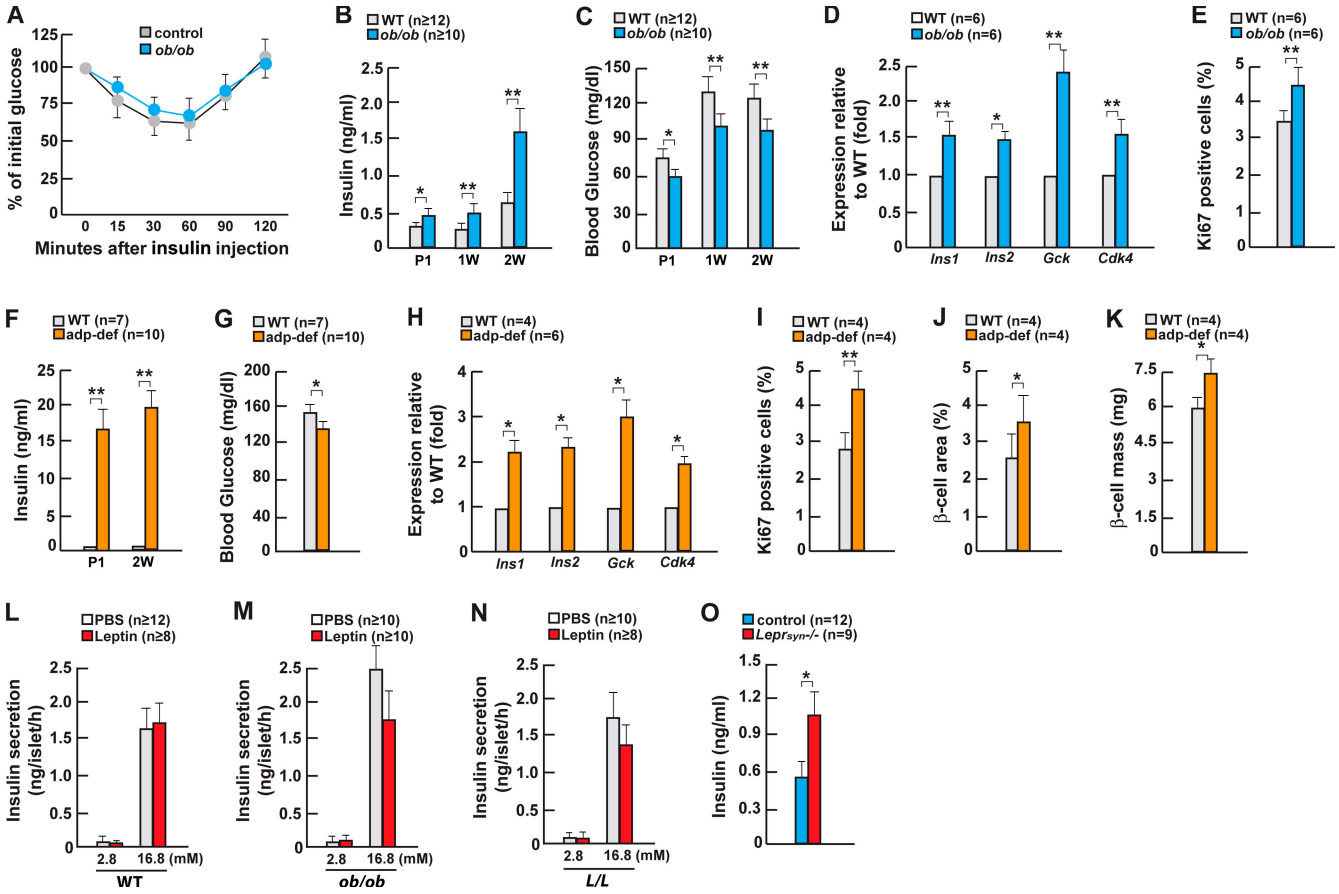

Figure 1. **Leptin regulates insulin secretion in part through the neuronal pathway.** (A) ITT in 2-wk-old *ob/ob* mice. (B and C) Serum insulin and blood glucose in *ob/ob* mice. (D) Gene expression in pancreas or islets of 2-wk-old *ob/ob* mice. (E) Quantification of insulin/Ki67 immunoreactive cells in islets of 2-wk-old *ob/ob* mice. (F and G) Serum insulin and blood glucose in adipocyte-deficient (adp-def) mice. (H) Gene expression in pancreas or islets of 2-wk-old adipocyte-deficient mice. (I–K) Quantification of insulin/Ki67 immunoreactive cells in islets, β-cell area, and β-cell mass of 2-wk-old adipocyte-deficient mice. (L–N) Glucose-stimulated insulin secretion by leptin in islets from WT, *ob/ob*, and *L/L* mice. (O) Serum insulin levels in 1-mo-old $Lepr_{syn}^{-/-}$ mice. Error bars indicate mean + SEM. *, P < 0.05; **, P < 0.01; P1, newborn; 1W, 1 wk old; 2W, 2 wk old. Control in O indicates *Synapsin-Cre* mice. In L–N, the concentration of glucose in the culture media is indicated in millimolars.

Leptin-dependent sympathetic regulation of insulin secretion occurs through the osteoblasts

Although isoproterenol can affect secretion of insulin by isolated islets (Iversen, 1973), two reasons led us to test the hypothesis that it was also through the osteoblasts that the sympathetic tone, under the control of leptin, regulates insulin secretion. One is the functional link existing between leptin, sympathetic tone, and bone mass (Takeda et al., 2002), and another one is the recently uncovered role of osteoblasts in regulating insulin secretion (Lee et al., 2007). To test this hypothesis, we deleted Adrβ2, the only adrenergic receptor expressed in osteoblasts, from these cells ($Adr\beta2_{osb}^{-/-}$ mice).

Consistent with our hypothesis, serum insulin levels were markedly higher in $Adr\beta2_{osb}^{-/-}$ mice than in WT mice (Fig. 2 G and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200809113/DC1). Accordingly, propranolol, a β blocker acting through Adrβ2, increased insulin serum levels in WT but not in $Adr\beta2_{osb}^{-/-}$ mice (Fig. 2 H). The increase in serum insulin levels developed in $Adr\beta2_{osb}^{-/-}$ mice in the absence of any evidence of insulin resistance as determined by insulin tolerance test (ITT), serum adiponectin level, and *Adiponectin* expression in fat (Fig. S2, F–H). This was specific of *Adrβ2* deletion because serum insulin levels were normal in $Adr\beta1^{-/-}$ and $Adr\alpha2A^{-/-}$ mice (unpublished data). $Adr\beta2_{osb}^{-/-}$ mice also had an 80–150% increase in *Insulin* and *Glucokinase* expression and exhibited postprandial low blood glucose levels (Fig. 2, I and J), and *Cdk4* expression was increased 50% in $Adr\beta2_{osb}^{-/-}$ mice (Fig. 2 J). It is worthwhile to note that these abnormalities were similar to the ones observed in *ob/ob* mice (compare with Fig. 1 D).

If sympathetic signaling in osteoblasts and leptin are in the same genetic pathway, genetic epistasis predicts that mice heterozygous for *Adrβ2* deficiency and *Leptin* deficiency should harbor the same defect in insulin secretion than the one observed in $Adr\beta2_{osb}^{-/-}$ and *ob/ob* mice. Indeed, although both $Adr\beta2_{osb}^{+/-}$ and *ob/+* mice had normal serum insulin levels, *ob/+*;$Adr\beta2_{osb}^{+/-}$ mice displayed a hyperinsulinemia and lower blood glucose level after feeding (Fig. 2, K and L). The severity of these two abnormalities was only slightly milder than in *ob/ob* mice (compare with Fig. 1, B and C). At the molecular level, *Insulin* expression was elevated to the same degree in *ob/ob* and *ob/+*;$Adr\beta2_{osb}^{+/-}$ mice (Fig. 2 M).

To add further credence to the notion that sympathetic signaling in osteoblasts contributes to the leptin regulation of

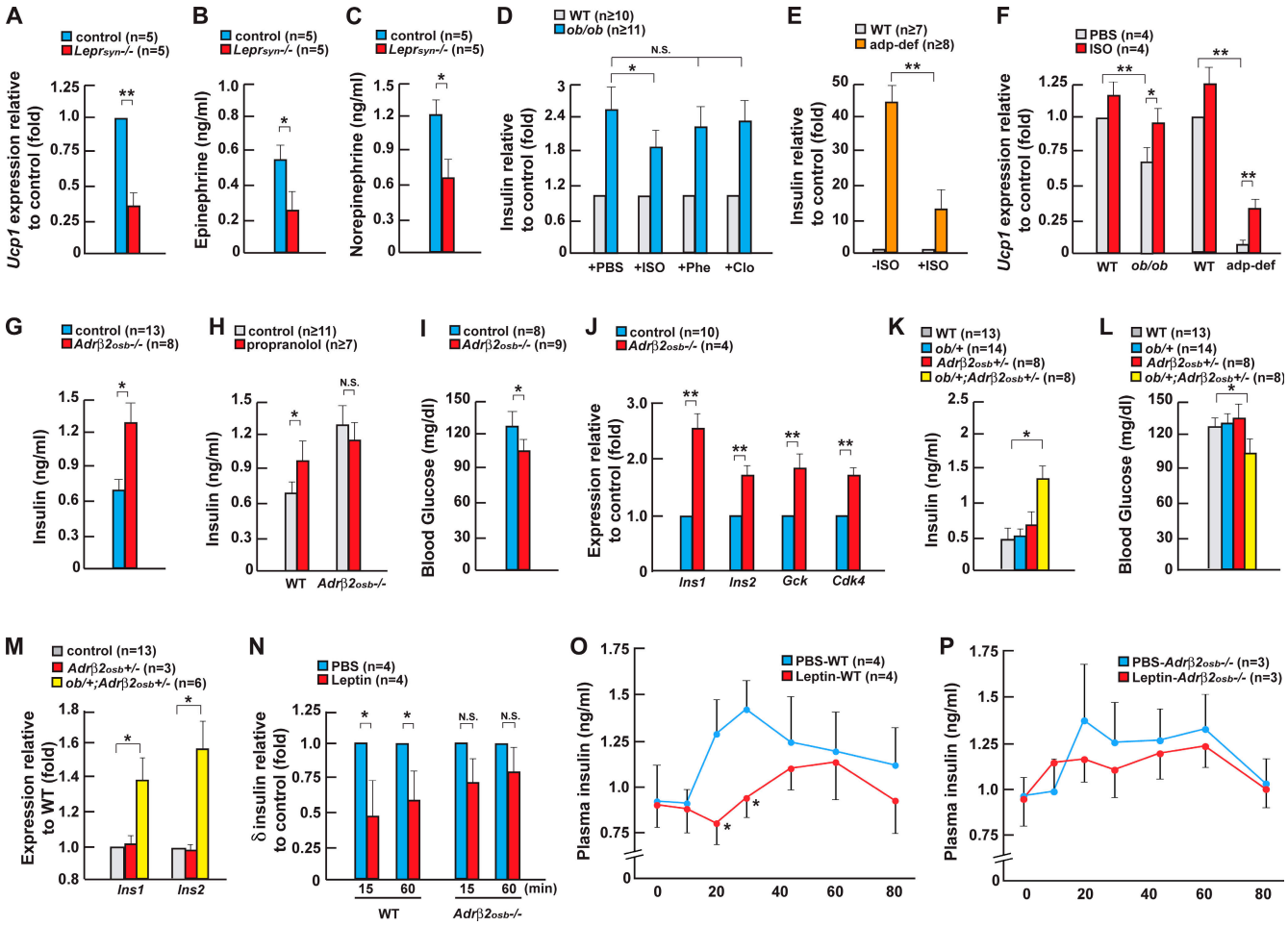

Figure 2. **Sympathetic signaling in osteoblasts regulates insulin expression and secretion.** (A) *Ucp1* expression in brown fat of $Lepr_{syn}^{-/-}$ mice. (B and C) Serum epinephrine and norepinephrine levels in $Lepr_{syn}^{-/-}$ mice. (D and E) Serum insulin levels in *ob/ob* and adipocyte-deficient (adp-def) mice after daily isoproterenol (+ISO), phenylephrine (+Phe), or clonidine (+Clo) injection for 2 wk. (F) *Ucp1* expression in brown fat of *ob/ob* and adipocyte-deficient mice after daily isoproterenol injection. (G) Serum insulin levels in 2-wk-old $Adr\beta2_{osb}^{-/-}$ mice. (H) Serum insulin levels in WT and $Adr\beta2_{osb}^{-/-}$ mice after daily propranolol injection for 2 wk. (I and J) Blood glucose levels and gene expression in islets of 2-wk-old $Adr\beta2_{osb}^{-/-}$ mice. (K–M) Serum insulin, blood glucose, and *Insulin* expression in the pancreas of 2-wk-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 wk. (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 wk. Error bars indicate mean + SEM. *, $P < 0.05$; **, $P < 0.01$. Control in A–C indicates *Synapsin-Cre* mice. Control in G–J and M indicates *α1(I)Collagen-Cre* mice.

insulin secretion, we performed two other experiments. Long-term (1 wk) intracerebroventricular (ICV) infusion of 4 ng/h leptin decreased glucose-stimulated insulin secretion in WT but not in $Adr\beta2_{osb}^{-/-}$ mice (Fig. 2 N). We also used a hyperglycemic clamp to assess glucose-stimulated insulin secretion in vivo. Leptin ICV infusion also decreased circulating insulin levels in WT but not $Adr\beta2_{osb}^{-/-}$ mice (Fig. 2, O and P). Collectively, these observations identify sympathetic signaling in osteoblasts as a significant mediator of leptin's regulation of insulin secretion.

The sympathetic tone in osteoblasts inhibits osteocalcin activity

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 (Lee et al., 2007; Ferron et al., 2008). Multiple observations indicate that the leptin-dependent sympathetic regulation of insulin secretion occurs in part by modulating osteocalcin bioactivity.

Esp expression was up-regulated fourfold by isoproterenol in osteoblasts, whereas expression of *Osteocalcin* and of other enzymes modifying it was not (Fig. 3 A). Accordingly, and although the serum level of total osteocalcin was normal (Fig. 3 B), the amount of osteocalcin binding to hydroxyapatite (HA) with low affinity, i.e., undercarboxylated and biologically active (Lee et al., 2007), was increased in both *ob/ob* and $Adr\beta2_{osb}^{-/-}$ mice (Fig. 3 C). 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 (unpublished data). Second, the low affinity fractions regulated *Insulin* expression in isolated islets, whereas the ones binding to HA with high affinity did not (Fig. 3 D).

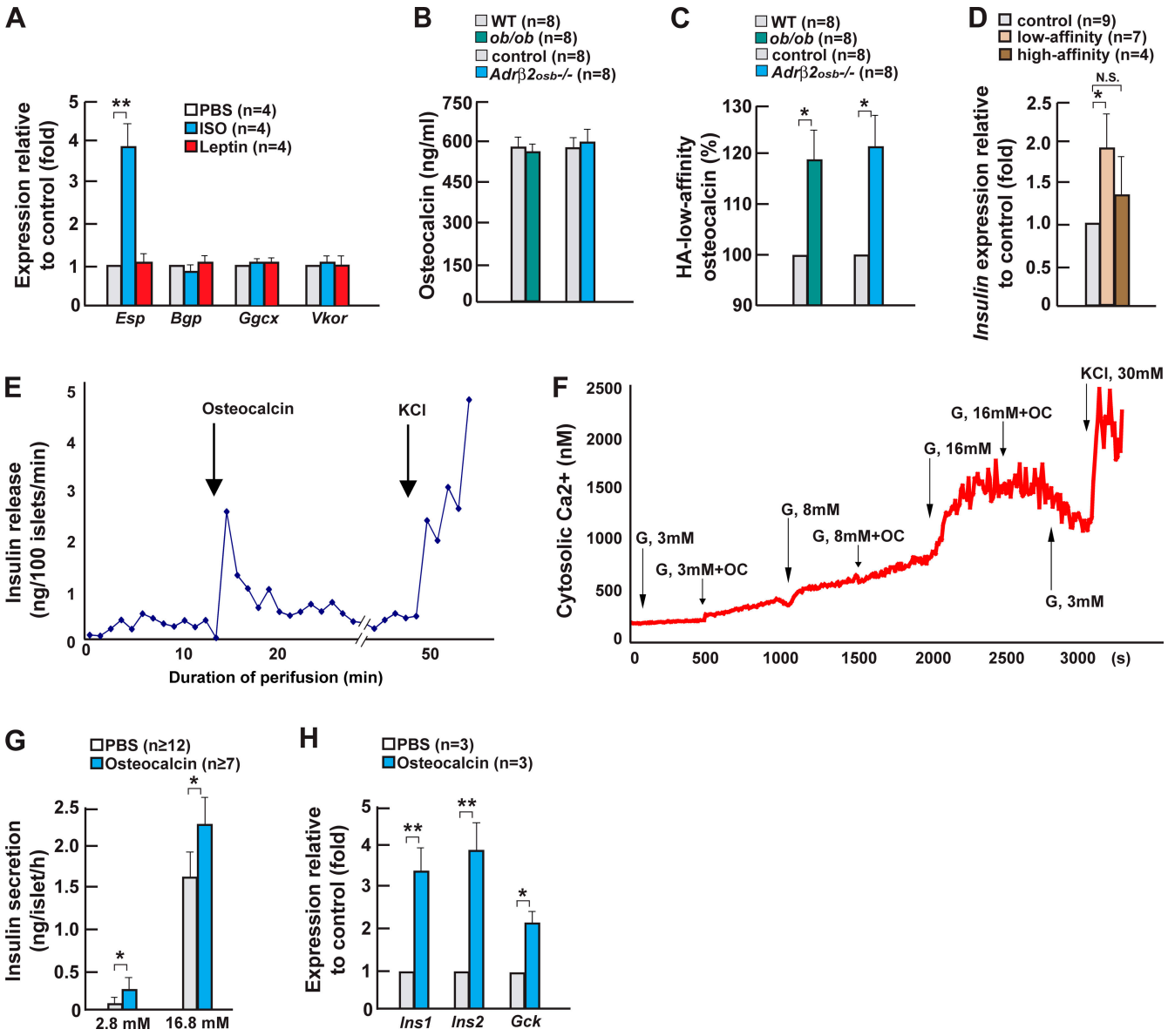

Figure 3. **Osteocalcin directly regulates insulin secretion in a Ca^{2+}-dependent manner.** (A) Gene expression in isoproterenol (ISO)- or leptin-treated osteoblasts. (B and C) Levels of total and uncarboxylated osteocalcin (HA low affinity) in *ob/ob* and $Adr\beta2_{osb}^{-/-}$ mice. (D) *Insulin* expression by HA affinity fraction in islets. (E) Perifusion of WT islets in the presence of 0.03 ng/ml osteocalcin under a 3-mM glucose condition. (F) Intracellular calcium imaging assay using WT islets in the presence of osteocalcin under the different concentrations of glucose. (G) Insulin secretion by osteocalcin in WT islets maintained in either low or high glucose. (H) Gene expression in osteocalcin-treated islets. Error bars indicate mean + SEM. *, $P < 0.05$; **, $P < 0.01$. Control in B and C indicates *α1(I)Collagen-Cre* mice. In G, the concentration of glucose in the culture media is indicated in millimolars.

Next, we tested whether osteocalcin regulates insulin secretion directly. In an islets perifusion assay, 0.03 ng/ml uncarboxylated osteocalcin was a powerful insulin secretagogue (Fig. 3 E) with a strong first phase and a sustained but lower second phase of insulin secretion in all experiments performed ($n = 3$). Cytosolic Ca^{2+} imaging was also performed on untreated and osteocalcin-treated WT mouse islets at 3-, 8-, and 16-mM glucose levels. 30 mM KCl was used as a positive control. At 3 mM glucose, osteocalcin initiated a steady increase of basal Ca^{2+} concentration, whereas at 8 and 16 mM glucose, osteocalcin notably enhanced the cytosolic Ca^{2+} concentration as compared with control untreated islets ($n = 3$; Fig. 3 F and Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200809113/DC1). Osteocalcin also augmented glucose-stimulated insulin secretion from isolated islets maintained in either low or high glucose concentrations (Fig. 3 G). In addition, expression of *Insulin* and *Glucokinase* in isolated islets (Fig. 3 H) was induced by osteocalcin in a time- and dose-dependent manner.

The relevance of osteocalcin in the leptin-dependent sympathetic regulation of insulin secretion was also verified in vivo. First, increasing osteocalcin bioactivity should worsen hyperinsulinemia in *ob/ob* mice. To test this hypothesis, we generated *ob/ob* mice lacking *Esp* (*ob/ob*;$Esp^{-/-}$ mice). *Ob/ob*;$Esp^{-/-}$ mice were twofold more hyperinsulinemic than *ob/ob* mice at 2 wk of age (Fig. 4 A). Remarkably, similar abnormalities were observed in *ob/ob* mice lacking only

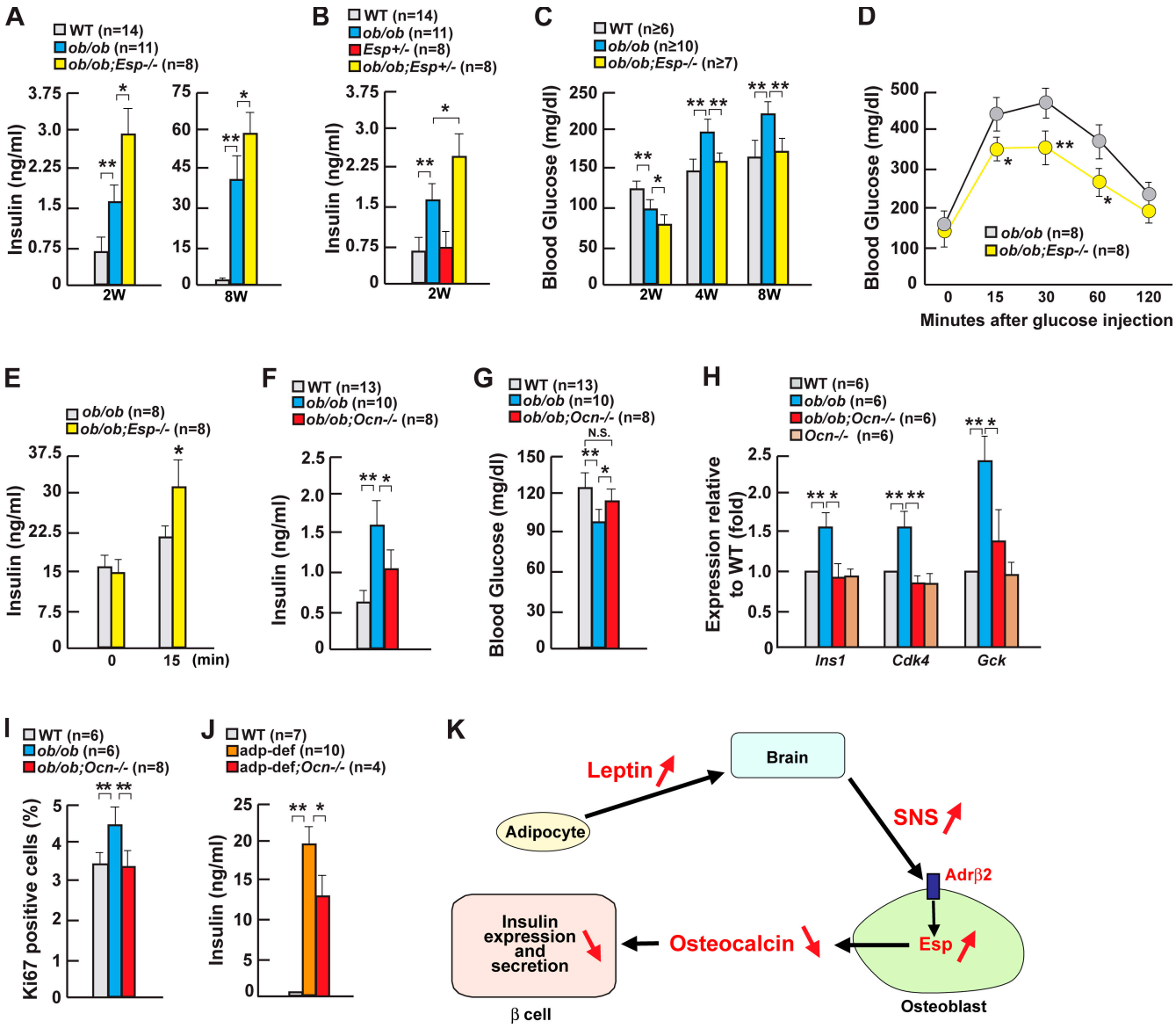

Figure 4. **Leptin regulates insulin secretion through osteoblasts.** (A) Serum insulin in 2- and 8-wk-old *ob/ob;Esp*$^{-/-}$ mice. (B) Serum insulin in 2-wk-old *ob/ob;Esp*$^{-/-}$ mice. (C) Blood glucose in 2–8-wk-old *ob/ob;Esp*$^{-/-}$ mice. (D) Glucose tolerance tests in 2-mo-old *ob/ob;Esp*$^{-/-}$ mice. (E) Serum insulin after glucose challenge in 2-mo-old *ob/ob;Esp*$^{-/-}$ mice. (F and G) Serum insulin and blood glucose in 2-wk-old *ob/ob;Ocn*$^{-/-}$ mice. (H) Gene expression in pancreas or islets of 2-wk-old *ob/ob;Ocn*$^{-/-}$ mice. (I) Quantification of insulin/Ki67-positive cell islets of 2-wk-old *ob/ob;Ocn*$^{-/-}$ mice. (J) Serum insulin in 2-wk-old adipocyte-deficient;*Ocn*$^{-/-}$ mice. adp-def, adipocyte deficient. (K) In the presence of leptin, sympathetic tone is high; thus, *Esp* expression in the osteoblast is high, and osteocalcin bioactivity is low. This contributes to the inhibition of insulin secretion by leptin. Error bars indicate mean + SEM. *, P < 0.05; **, P < 0.01. 2W, 2 wk old; 4W, 4 wk old; 8W, 8 wk old; SNS, sympathetic nervous system.

one allele of *Esp* (Fig. 4 B). *Ob/ob*;*Esp*$^{-/-}$ mice also had low blood glucose levels and remained significantly less glucose intolerant than *ob/ob* mice until 8 wk of age, thus illustrating the importance of this pathway in adult mouse physiology (Fig. 4, A, C, and D). An 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. 4 E). Insulin sensitivity, as measured by hyperinsulinemic-euglycemic clamps, was similar in *ob/ob*;*Esp*$^{-/-}$ and *ob/ob* mice (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200809113/DC1).

Second, *Osteocalcin* inactivation decreased serum insulin levels 50% and normalized blood glucose levels in *ob/ob* mice (*ob/ob*;*Osteocalcin*$^{-/-}$; Fig. 4, F and G). Expression of *Insulin*, *Glucokinase*, and *Cdk4* was decreased 50–100% in *ob/ob*;*Osteocalcin*$^{-/-}$ mice compared with *ob/ob* mice, and β-cell proliferation was normalized in *ob/ob*;*Osteocalcin*$^{-/-}$ mice (Fig. 4, H and I). The similarity of correction of hyperinsulinemia in *ob/ob*;*Osteocalcin*$^{-/-}$ mice and in *ob/ob* mice treated with the sympathomimetic isoproterenol supports the view that osteocalcin is in vivo a significant mediator of the leptin-dependent sympathetic regulation of insulin secretion. *Osteocalcin* inactivation also decreased serum insulin levels by half in adipocyte-deficient mice (Fig. 4 J). Collectively, the results presented in this study support the hypothesis that the leptin-dependent sympathetic inhibition of insulin secretion occurs in part via the osteoblast and acts by modulating osteocalcin activity.

Our results reveal that leptin inhibits the endocrine function of the skeleton. Indeed, to a substantial extent, leptin's inhibition of insulin secretion relies on a three-step cascade, including (1) leptin's up-regulation of sympathetic tone, (2) sympathetic enhancement of *Esp* expression in osteoblasts, and (3) decrease in osteocalcin bioactivity. Osteocalcin acts in this context primarily by regulating insulin expression and secretion. These results do not contradict that leptin acts locally on β cells to inhibit insulin secretion (Covey et al., 2006; Morioka et al., 2007); rather, they reveal an additional pathway whereby leptin fulfills this function. Beyond linking two secreted molecules, i.e., leptin and osteocalcin, these observations establish that the metabolic connection between osteoblasts and adipocytes is tighter than originally thought (Karsenty, 2006; Rosen, 2008). This cross talk includes an important functional component because osteoblasts mediate a significant part of one of the endocrine functions on adipocytes. This unexpected functional relationship between adipocytes, neurons, sympathetic tone, and osteoblasts illustrates in vivo the importance of the skeleton in the regulation of glucose homeostasis and provides an example of how critical the interplay is between multiple organs in the establishment and regulation of major physiological functions in vertebrates.

Materials and methods

Animals, treatments, and surgical procedures

Adrα2A$^{-/-}$ (Q. Wang, University of Alabama at Birmingham, Birmingham, AL), *Osteocalcin*$^{-/-}$, *Esp*$^{-/-}$, *Lepr-flox/flox*, and *α1(I)Collagen-Cre* mice were previously described (Altman et al., 1999; Dacquin et al., 2002; Lee et al., 2007; van de Wall et al., 2008). Osteoblast-specific *Adrβ2*-deficient (*Adrβ2*$_{osb}^{-/-}$) mice were generated through homologous recombination in embryotic stem cells and through the use of *α1(I)Collagen-Cre* mice. B6.V-*Lep*^{ob}/J and FVB-Tg(AZIP/F)1Vsn/J mice were purchased from The Jackson Laboratory. 3 mg/kg isoproterenol was injected i.p. once daily for 2 wk. For ICV infusion, a 28-gauge cannula (brain infusion kit II; Alza) was implanted into the third ventricle as previously described, infusing human leptin (Sigma-Aldrich) at 4 ng/h for 7 d (Ducy et al., 2000). The cannula was connected to an osmotic pump (Alza) placed in the dorsal subcutaneous space of the animal. Genotyping was performed by PCR analysis of genomic DNA. All procedures involving animals were approved by the Institutional Animal Care and Use Committee and conform to the relevant regulatory standards.

Metabolic studies

For glucose tolerance tests, 0.625 or 2 g/kg glucose was injected i.p. after an overnight fast, and blood glucose was monitored using blood glucose strips and the Accu-Check glucometer (Roche) at the indicated times. For ITT, mice were injected i.p. with 0.25 or 0.5 U/kg insulin, and blood glucose levels were measured at the indicated times. ITT data are presented as a percentage of initial blood glucose concentration. Hyperinsulinemic-euglycemic clamps and hyperglycemic clamps were performed at the Pennsylvania State Diabetes and Obesity Mouse Phenotyping Center. In brief, *ob/ob*, *ob/ob;Esp*$^{-/-}$, and WT littermate mice (n = 4 for each group) were fasted overnight, and a 2-h hyperinsulinemic (2.5 mU/kg/min)-euglycemic clamp was performed after intravenous administration of [3-^{3}H] glucose and 2-deoxy-D-[1-^{14}C] glucose as previously described (Kim et al., 2004). *Adrβ2*$_{osb}^{-/-}$ and control mice (n = 4 for each group) were fasted overnight, and a 2-h hyperglycemic clamp was conducted with a variable infusion of 20% glucose to maintain hyperglycemia at ∼300 mg/dl. Blood samples were taken at 10–20-min intervals for the measurement of plasma insulin levels (Cho et al., 2007).

Islet perifusion

Islet perifusion was performed as described previously (Gao et al., 2007). In brief, islets were isolated from WT mice using standard collagenase digestion followed by purification through a Ficoll gradient. 100 islets were hand picked under a light microscope and placed into a perifusion chamber (Millipore). A computer-controlled high performance liquid chromatography system (625 LC; Waters Corporation) allowed programmable rates of flow and concentration of the appropriate solutions held in a 37°C water bath. Islets were perifused with Krebs bicarbonate buffer to reach baseline hormone secretion values before the addition of the appropriate secretagogues. Samples were collected at regular intervals with a fraction collector (Waters Corporation).

Ca^{2+} imaging

Cytosolic Ca^{2+} imaging was performed as described previously (Gao et al., 2007). Mouse islets cultured for 3–4 d in 10 mM glucose were loaded with 1 mmol/liter fura 2-AM (Invitrogen) during a 40-min pretreatment at 37°C in Krebs buffer, transferred to the perifusion chamber, and placed on the homeothermic platform of an inverted microscope (Axio Observer Z1; Carl Zeiss, Inc.). Islets were perfused with Krebs buffer at 37°C while various treatments were applied. The microscope was used with a 40× oil immersion objective. Intracellular Ca^{2+} was determined by the ratio of the excitation of fura 2-AM at 334 and 380 nm. Emission was measured at 520 nm with a charge-coupled device camera (Attofluor; Invitrogen) and calibrated using the Ratio Vision software (Attofluor; Invitrogen).

In vitro glucose-stimulated insulin secretion

For assay of insulin release (Kitamura et al., 2001), five islets were manually selected, incubated in Krebs-Ringer solution, and stimulated at 37°C with various concentrations of glucose in the presence or absence of osteocalcin for 1 h. The islets were collected by centrifugation, and the supernatant was assayed for insulin content by ELISA (Crystal Chem).

Cell and molecular studies

Primary osteoblasts were prepared from calvaria of 5-d-old pups as previously described (Takeda et al., 2002). Real-time PCR was performed on Dnase I–treated total RNA converted to cDNA using iTaq SYBR Green Supermix with ROX (6-carboxy-X-rhodamine; Bio-Rad Laboratories) on an MX3000 instrument; β-actin amplification was used as an internal reference for each sample.

Biochemistry

Serum levels of insulin (Crystal Chem) and c-peptide (Yanaihara Institute) were measured using commercial kits. For quantification of uncarboxylated levels of osteocalcin, sera were incubated with HA slurry for 1 h. The quantity of osteocalcin present in the unbound fraction and in the fractions eluted at 0.02 and 1 M sodium phosphate, pH 6.8, was measured by an immunoradiometric assay (Immunotopics).

Histology

Pancreas was fixed in 10% neutral formalin, embedded in paraffin, and sectioned at 5 µm; sections were stained with hematoxylin and eosin. Immunohistochemistry was performed using rabbit antiinsulin (Santa Cruz Biotechnology, Inc.) and mouse anti-Ki67 (Vector Laboratories) antibodies. β-cell area represents the surface positive for insulin immunostaining divided by the total pancreatic surface. β-cell mass was calculated as β-cell area multiplied by pancreatic weight as described previously (Lee et al., 2007).

Statistical analyses

Results are given as means ± SEM. Statistical analyses were performed using unpaired two-tailed Student's t or analysis of variance tests.

Online supplemental material

Fig. S1 shows the characterization of *ob/ob* and adipocyte-deficient mice. Fig. S2 shows the strategy for generation of conditional *Adrβ2*-deficient mice and the characterization of *Adrβ2*$_{osb}^{-/-}$ mice. Fig. S3 shows the intracellular calcium imaging performed on untreated WT mice islets at different concentrations of glucose. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200809113/DC1.

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