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# Bone Marrow (BM) Transplantation Promotes β-Cell Regeneration after Acute Injury through BM Cell Mobilization

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**There is controversy regarding the roles of bone marrow (BM)-derived cells in pancreatic β-cell regeneration. To examine these roles *in vivo*, mice were treated with streptozotocin (STZ), followed by bone marrow transplantation (BMT; lethal irradiation and subsequent BM cell infusion) from green fluorescence protein transgenic mice. BMT improved STZ-induced hyperglycemia, nearly normalizing glucose levels, with partially restored pancreatic islet number and size, whereas simple BM cell infusion without preirradiation had no effects. In post-BMT mice, most islets were located near pancreatic ducts and substantial numbers of bromodeoxyuridine-positive cells were detected in islets and ducts. Importantly, green fluorescence protein-positive, *i.e.* BM-derived, cells were detected around islets and were CD45 positive but not insulin positive. Then to examine whether BM-derived cell mobilization contributes to this process, we used *Nos3*$^{-/-}$ mice as a model of impaired BM-derived cell mobilization. In streptozotocin-treated *Nos3*$^{-/-}$ mice, the effects of BMT on blood glucose, islet number, bromodeoxyuridine-positive cells in islets, and CD45-positive cells around islets were much smaller than those in streptozotocin-treated *Nos3*$^{+/+}$ controls. A series of BMT experiments using *Nos3*$^{+/+}$ and *Nos3*$^{-/-}$ mice showed hyperglycemia-improving effects of BMT to correlate inversely with the severity of myelosuppression and delay of peripheral white blood cell recovery. Thus, mobilization of BM-derived cells is critical for BMT-induced β-cell regeneration after injury. The present results suggest that homing of donor BM-derived cells in BM and subsequent mobilization into the injured periphery are required for BMT-induced regeneration of recipient pancreatic β-cells. (*Endocrinology* 148: 2006–2015, 2007)**

SEVERAL LINES OF evidence indicate that bone marrow (BM)-derived cells are capable of transdifferentiating into various cell types, including endothelial cells, arterial smooth muscle cells, myoblasts, myocardium, and epithelia of the gastrointestinal tract (1–6). In the field of regenerative medicine for diabetes treatment, BM cells are seen as promising pancreatic β-cell sources (7–9). However, whether BM cells can transdifferentiate into β-cells and/or stimulate β-cell differentiation is controversial.

A previous study (10) showed that BM-derived cells can directly transdifferentiate into β-cells. In that report, 4–6 wk after BM transplantation (BMT; *i.e.* lethal irradiation of recipient mice and subsequent BM cell infusion from other mice), donor BM-derived insulin-positive cells were detected in 1.7–3% of pancreatic islet cells. However, in subsequent similar studies (11–13), very few or no donor BM-derived insulin-positive cells were detected in recipient islets, suggesting that if direct transdifferentiation from BM-derived cells into β-cells occurs, it would involve only a very small percentage of cells. BM-derived cells also reportedly initiate recipient β-cell regeneration rather than directly transdifferentiating into β-cells (14). In that study, BMT increased recipient β-cells with the appearance of donor-derived endothelial cells in the pancreas, resulting in improvement of hyperglycemia in streptozotocin (STZ)-induced diabetic mice. Other studies also demonstrated that BMT improves hyperglycemia in diabetic animals such as STZ-treated mice (15) and rats (16), E2f1/E2f2 mutant mice (17), and KKAy mice (18). However, several studies obtained contradictory results, *i.e.* no improvement in hyperglycemia after BMT (12, 19). Whether BMT promotes β-cell regeneration and improves hyperglycemia in diabetic mice and, if so, how β-cells are regenerated remains essentially unknown. Herein we attempted to address these questions.

First, we observed that BMT, but not simple BM cell infusion without preirradiation, restored islet numbers and improved hyperglycemia in STZ-treated mice. Donor-derived cells were detected around post-BMT islets and were

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* Y.H. and T.O. contributed equally to this work.

Abbreviations: BM, Bone marrow; BMT, BM transplantation; BrdU, bromodeoxyuridine; eNOS, endothelial nitric oxide synthase; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GFP, green fluorescence protein; MMP, matrix metalloproteinase; PECAM, platelet endothelial cell adhesion molecule; sKitL, soluble kit-ligand; STZ, streptozotocin; WBC, white blood cell.

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CD45 (pan-hematopoietic marker) positive, suggesting that mobilization of BM-derived cells to the pancreas induces β-cell regeneration. To examine this hypothesis, we performed BMT experiments using endothelial nitric oxide synthase (eNOS)-deficient (*Nos3*$^{-/-}$) mice, in which mobilization of BM-derived cells after myelosuppression is impaired (20). In STZ-treated *Nos3*$^{-/-}$ mice, BMT effects on β-cell regeneration and improvement of hyperglycemia were very limited. Thus, BM-derived cell mobilization is apparently involved in BMT-induced β-cell regeneration after acute injury.

## Materials and Methods

### *Animals*

C57BL/6J mice were purchased from Clea Japan, Inc. (Tokyo, Japan). Green fluorescent protein (GFP) transgenic mice with the C57BL/6J background were kindly provided by Dr. M. Okabe (Osaka University, Osaka, Japan) (21). Enhanced GFP is under transcriptional control of the chicken β-actin promoter and the cytomegalovirus enhancer in this strain, resulting in high-level expression in most tissues. *Nos3*$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Age- and sex-matched wild-type (*Nos3*$^{+/+}$) littermates served as controls. These animals were generated and have been maintained with a C57BL/6J background by backcrossing of hemizygous carriers to C57BL/6J for more than six generations. Mice were housed in an air-conditioned environment, with a 12-h light, 12-h dark cycle, and fed a regular unrestricted diet. Hyperglycemia was induced by ip infusion of 35 mg/kg body weight STZ (Sigma-Aldrich, St. Louis, MO) daily for 8 d [modification of method reported by Wang *et al.* (22)]. STZ was solubilized in citrate sodium buffer (pH 4.5) and injected, within 15 min after preparation, into 6-wk-old mice. All animal experiment procedures were approved by our Institutional Review Board, Tohoku University School of Medicine, and conducted according to institutional guidelines for animal experiments.

### *Measurements*

Blood glucose was measured after a 10-h fast and assayed using Antsense II (Horiba Industry, Kyoto, Japan). Plasma insulin was determined with an ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan). Insulin content was measured as described previously (23).

### *BMT*

BM cells were flushed in bulk from the medullary cavities of femurs and tibias. BM donors were young (6 wk old) sex-matched GFP transgenic mice and *Nos3*$^{-/-}$ or *Nos3*$^{+/+}$ mice. Recipient mice were lethally irradiated (10 Gy) and reconstituted a single iv infusion of $2 \times 10^6$ BM cells, from donor mice through the tail vein. Tissues were analyzed 30–40 d after BMT. The percentage of GFP-positive cells among recipient BM cells was determined by fluorescence-activated cell sorting (FACS), using a FACS Caliber with CellQuest software (BD PharMingen, Franklin Lakes, NJ).

### *Bromodeoxyuridine (BrdU) in situ detection*

To identify proliferating cells in the pancreas, BrdU was injected according to the BrdU *in situ* detection kit protocol (BD Bioscience, San Jose, CA). Mice were injected ip with 1 mg BrdU 24 h before pancreas extraction at 0, 3, 7, 10, 15, or 25 d after BMT. The labeled cells were immunostained with anti-BrdU antibody. To calculate numbers of islets and cells per islet and the percentage of BrdU-positive cells among islet cells, we microscopically examined the whole pancreas in 30-μm sections and counted the numbers of islets, islet cells, and BrdU-positive nuclei in islets.

### *Immunohistochemistry*

Mouse pancreases were excised and fixed overnight in 10% paraformaldehyde. Fixed tissues were processed for paraffin embedding and 3-μm sections were prepared. The streptavidin-biotin method was performed with a Histofine streptavidin-biotin-PO kit (Nichirei, Tokyo, Japan) for immunostaining using antibody against insulin (Sigma-Aldrich) or GFP (Santa Cruz Biotechnology, Santa Cruz, CA). Slides were deparaffinized and immediately exposed to the blocking solution. Sections were incubated for 18 h at 4 C with antibody against human insulin or GFP diluted 1:1000 in PBS. Slides were incubated with the biotinylated IgG for 1 h and then peroxidase-conjugated streptavidin for 30 min at room temperature. Finally, immunoreactivity was visualized by incubation with a substrate solution containing 3,3′-diaminobenzidine tetrahydrochloride. For double staining of insulin and BrdU, the streptavidin-peroxidase method was applied, followed by incubation with Simple stain 3-amino-9-ethyl carboxazole solution (Nichirei).

### *Fluorescent immunohistochemistry*

For double staining of insulin with glucagon, keratin/cytokeratin, or CD45, the 3-μm sections of paraffin-embedded pancreases were incubated overnight with the respective antibodies at 4 C. Antibodies against insulin, glucagon (Dako Corp., Carpinteria, CA), keratin/cytokeratin (Nichirei, and CD45 (Santa Cruz Biotechnology) were diluted 1:1000 in PBS. For platelet endothelial cell adhesion molecule (PECAM)-1 staining, sections were immunostained with rat anti-CD31 (1:10; BD Biosciences). Labeled cells were visualized with a biotin-conjugated secondary antibody with streptavidin, TX red conjugate (Vector Laboratories, Burlingame, CA). For double staining of insulin with glucagon or keratin/cytokeratin, the sections were incubated for 1 h at room temperature in a mixture of Alexa Fluor 488 goat chicken antimouse IgG (Molecular Probes, Eugene, OR) diluted 1:100 and Alexa Fluor 594 donkey antirabbit diluted 1:50 in PBS. For double staining of insulin and CD45, the sections were incubated in a mixture of Alexa Fluor 488 chicken antimouse IgG and Alexa Fluor 546 goat antirabbit IgG diluted 1:1000 in PBS. Sections were observed under a fluorescence microscope, LSM 5 PASCAL (Carl Zeiss, Oberkochen, Germany) and the image was analyzed using the PASCAL system.

### *Statistical analysis*

Data are expressed as means ± SE. Differences between experimental groups were evaluated using the unpaired Student's *t* test for several independent observations. $P < 0.05$ was considered significant.

## Results

### *Recipient BM was replaced with donor cells after irradiation followed by BM cell infusion but not after simple BM cell infusion without preirradiation*

Six-week-old C57BL/6J mice were given STZ daily for 8 d, followed by lethal irradiation and subsequent infusion of BM cells (STZ+BMT mice). In these experiments, BM cells were obtained from GFP transgenic mice (Fig. 1A). A group of STZ-treated mice was simply infused with the same number ($2 \times 10^6$) of BM cells without preirradiation (STZ+BM-infused mice). First, we confirmed replacement of recipient BM with that of donor mice using fluorescence microscopy and FACS analysis. As shown in Fig. 1B, there were no GFP-positive cells in the BM of C57BL/6J mice, whereas nearly all BM cells from GFP mice were GFP positive. BM cells of STZ+BMT mice showed high donor chimerism, indicating the recipient BM to have essentially been replaced with donor BM cells. In contrast, STZ+BM-infused mice had no donor-derived GFP cells in their BM, suggesting that preirradiation is necessary for BM replacement.

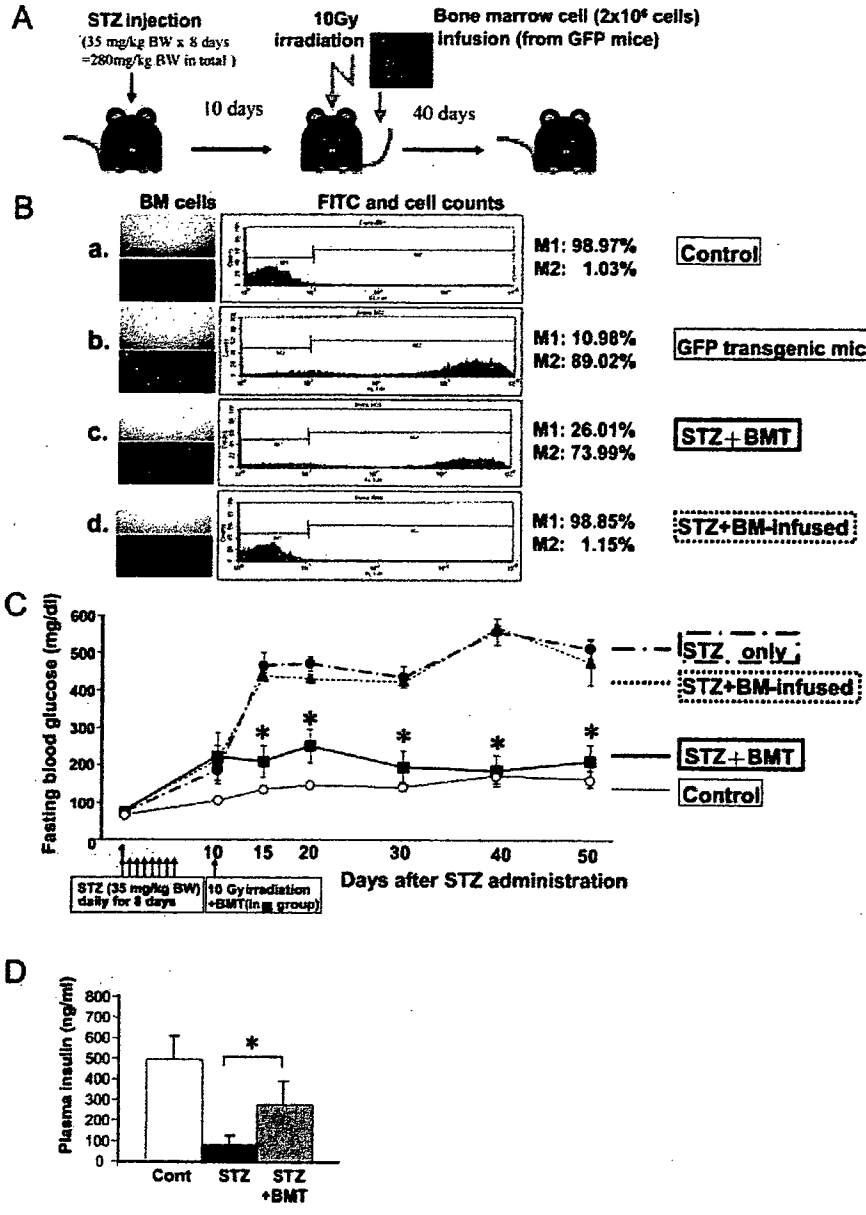


FIG. 1. BMT in STZ-treated mice. A, Experimental protocol. B, Bone marrow chimerism. Bright field (*left upper panels*), FITC (*left lower panels*), and representative FACS analyses of bone marrow chimerism from C57BL/6J mice (a), GFP transgenic mice (b), STZ-treated mice receiving lethal irradiation and BMT from GFP transgenic mice (c), and STZ-treated mice infused with BM cells of GFP transgenic mice, without preirradiation (d) (*right panels*) are shown. C, Fasting blood glucose of STZ-treated mice with or without BMT. ▲, STZ-treated mice without BMT (hyperglycemic control); ■, STZ-treated mice receiving BMT (lethal irradiation and subsequent BM cell infusion from GFP transgenic mice); ●, STZ-treated mice infused with BM cells of GFP transgenic mice, without preirradiation; ○, mice with neither STZ nor BMT (normoglycemic control). *, $P < 0.05$ for ■, compared with ▲ group (n = 5–6 in each group). D, Fasting plasma insulin on d 40. Cont, Mice with neither STZ nor BMT (normoglycemic control); STZ, STZ-treated mice without BMT (hyperglycemic control); STZ+BMT, STZ-treated mice receiving BMT. *, $P < 0.05$ for STZ+BMT, compared with STZ group (n = 5–6 in each group).

*BMT, but not simple BM cell infusion without preirradiation, improved hyperglycemia in STZ-treated mice*

As shown in Fig. 1C, STZ-treated mice receiving neither irradiation nor BM cell infusion (hyperglycemic controls) showed markedly higher fasting blood glucose than mice without STZ treatment (normoglycemic controls). Notably, blood glucose levels of STZ+BMT mice were significantly lower than those of hyperglycemic controls. Forty days after the first STZ administration, blood glucose levels of STZ+BMT mice were similar to those of normoglycemic controls. However, blood glucose levels of STZ+BM-infused mice did not decrease, instead remaining similar to those of hyperglycemic controls for 50 d after STZ administration. We additionally examined the effects of BMT, performed 30 d after STZ treatment. This late BMT did not significantly decrease blood glucose levels (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Together, these findings suggest that BMT improves hyperglycemia after acute injury of pancreatic β-cells with STZ treatment.

Next, we measured fasting plasma insulin levels on d 40 (Fig. 1D) in STZ+BMT mice. STZ administration markedly decreased plasma insulin levels, whereas BMT partially but significantly restored these levels by d 40.

*STZ administration followed by BMT increased pancreatic islets in the vicinity of pancreatic ducts*

We histologically analyzed pancreatic islets in the four groups. With hematoxylin-eosin staining on d 35, islet number and size were markedly decreased in hyperglycemic (Fig. 2A, b and f), as compared with normoglycemic (Fig. 2A, a and e), controls. Whereas simple BM infusion without preirradiation did not reverse the diminished number and size of

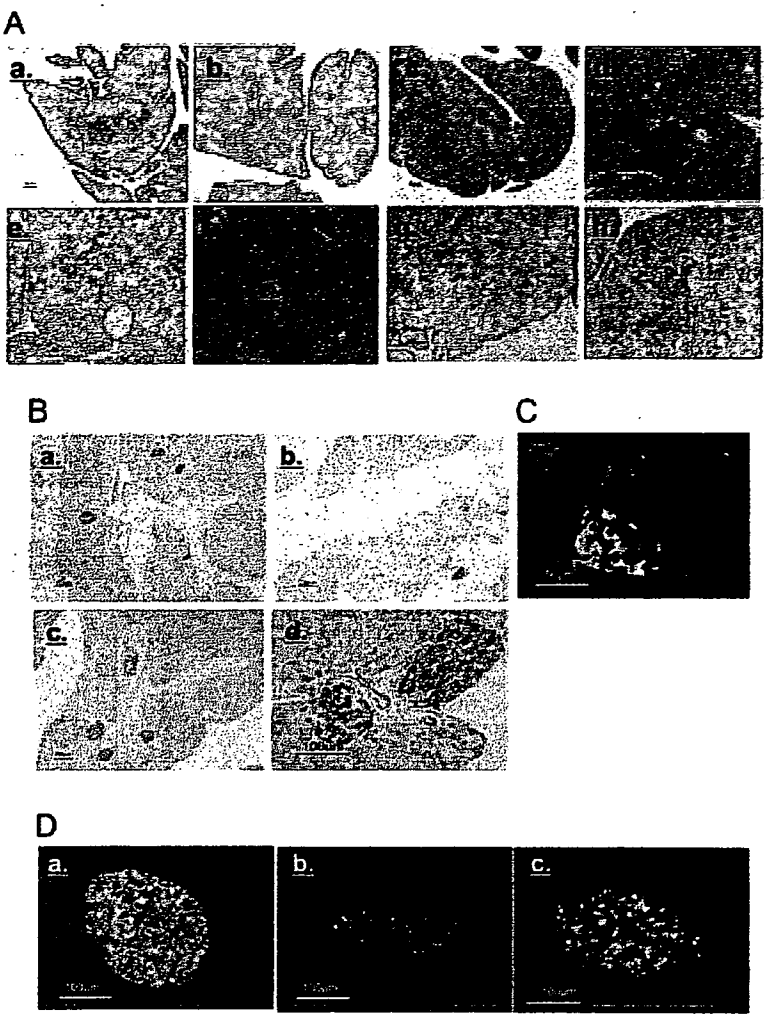


FIG. 2. Pancreatic islets of STZ-treated mice receiving subsequent BMT. A, Hematoxylin-Eosin staining of pancreases on d 35. Pancreases from normoglycemic control mouse (a and e), hyperglycemic control mouse (b and f), STZ-treated mouse simply infused with BM cells without preirradiation (c and g), and STZ-treated mouse receiving lethal irradiation and BMT (d and h). a–d, Magnification, ×40; e–h, ×100. B, Antiinsulin immunostaining of pancreases. Pancreases from normoglycemic control mouse (a), hyperglycemic control mouse (b), and STZ-treated mouse receiving BMT (c and d). a–c, Magnification, ×40; d, ×200. C, Double immunostaining of pancreas with antiinsulin and antikeratin/cytokeratin antibodies. *Green* indicates insulin-positive and *red* keratin/cytokeratin-positive cells, *i.e.* pancreatic ductal epithelium. D, Double immunostaining of pancreases with antiinsulin and antiglucagon antibodies. Pancreases from normoglycemic control mouse (a), hyperglycemic control mouse (b), and STZ-treated mouse receiving BMT (c). In C and D, to avoid overlapping staining of GFP with FITC, BM cells obtained from wild-type C57BL/6J mice, but not from GFP transgenic mice, were transplanted. Representative histological findings among six independent experiments are presented.

islets (Fig. 2A, c and g), islet number and size were both restored in STZ+BMT mice (Fig. 2A, d and h). Several islet populations were enlarged as compared with those in normoglycemic controls (Fig. 2A, a and e *vs.* d and h).

Antiinsulin staining of pancreatic specimens is shown in Fig. 2B. In hyperglycemic controls, insulin-positive cells were markedly diminished (Fig. 2B, b) as compared with normoglycemic controls (Fig. 2B, a). In contrast, in STZ+BMT mice, islet numbers were restored and sizes varied with some being enlarged (Fig. 2B, c). Notably, in the large view (Fig. 2B, d), a major population of insulin-positive cells in STZ+BMT mice is located in the vicinity of pancreatic ducts, which were stained with antikeratin/cytokeratin antibody (Fig. 2C).

Next, we performed double immunostaining using antibodies against insulin and glucagon. In immunofluorescent experiments (Figs. 2, C and D), to avoid overlapping staining of GFP with fluorescein isothiocyanate (FITC), BM cells obtained from wild-type C57BL/6J mice, but not GFP transgenic mice, were transplanted. Compared with islets of normal and STZ-treated mice (Fig. 2D, a and b), islets in STZ+BMT mice exhibited normal architecture with slightly fewer β-cells surrounded by α-cells (Fig. 2D, c).

To exclude the possibility that irradiation suppresses inflammation in response to STZ and prevents β-cell injury, STZ-treated mice were exposed to lethal irradiation (10 Gy) without subsequent BM cell infusion. Lethal irradiation alone did not lower blood glucose in STZ-treated mice. Pancreatic islets were diminished in size, as in hyperglycemic controls, 9 d after irradiation (mice died 10–14 d after lethal irradiation without BMT in our experiment; data not shown). Next, to examine prolonged effects of irradiation, mice were sublethally irradiated (5 Gy). Sublethal irradiation alone likewise did not significantly improve hyperglycemia in STZ-treated mice (data not shown), suggesting that irradiation does not exert protective effects against STZ-induced β-cell injury.

To further examine whether these islets in STZ+BMT mice were regenerated or only protected from STZ injury, BrdU staining was performed. In islets of normoglycemic (Fig. 3A) and hyperglycemic (Fig. 3B) controls, there were very few BrdU-positive cells. In contrast, islets of STZ+BMT mice (10 d after BMT) contained substantial numbers of BrdU-positive cells in and around islets, and some were detected among the pancreatic ductal cells (Fig. 3C). In other sections as well, islets containing BrdU-positive cells were mostly located near ducts and blood vessels. Most BrdU-positive cells in islets were insulin positive, whereas those outside the islets, mostly in the ductal structure, did not express insulin.

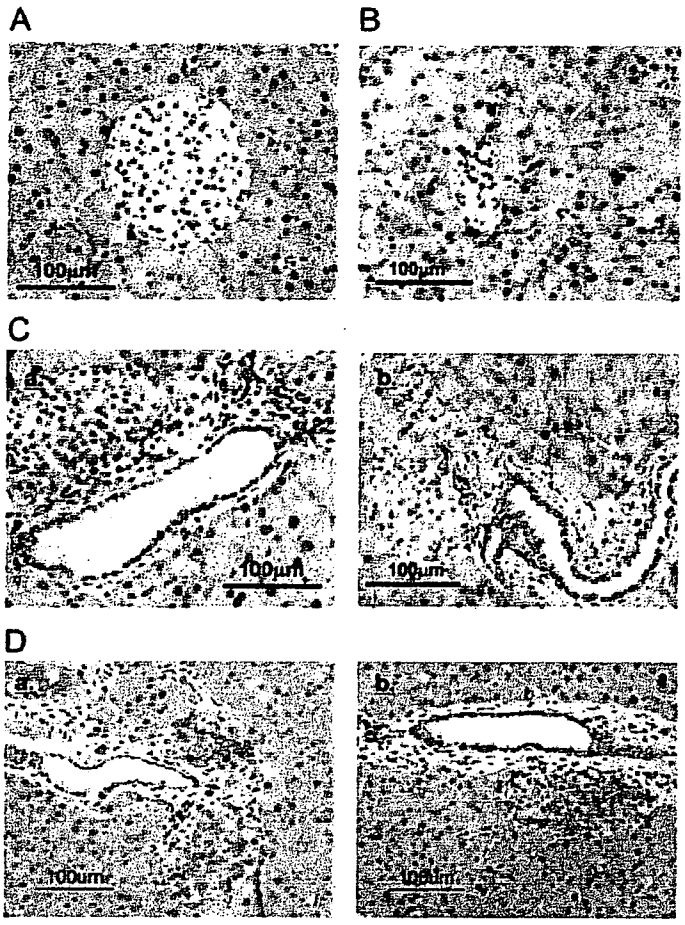


FIG. 3. BrdU-positive proliferating cells in pancreases of STZ-treated mice receiving subsequent BMT. A–C, Pancreases from normoglycemic control mouse (A), hyperglycemic control mouse (B), and STZ-treated mice receiving BMT (C) (10 d after BMT). *Brown cells* are BrdU positive. D, Double immunostaining of pancreases from STZ-treated mice receiving BMT (10 d after BMT) with antiinsulin and anti-BrdU. *Brown* and *red cells* are BrdU and insulin positive, respectively.

(Fig. 3D). Given reports that pancreatic stem/progenitor cells exist among ductal cells (24–26), BMT after STZ treatment might stimulate the generation of new islets from ductal progenitor cells as well as proliferation of β-cells in this model.

We also quantitatively examined the time courses of islet numbers, cell number per islet and percentage of BrdU-positive cells among islet cells after BMT (Table 1). Although decreased by STZ, islet number was significantly increased 10 and 15 d after BMT. The peak islet number was greater than in normoglycemic control mice by 47%. The islet number and percentage of BrdU-positive cells among islet cells were also increased through 10 d after BMT and then fell to normoglycemic control levels. These findings clearly indicate that BMT induces β-cell regeneration, resulting in pancreatic islet restoration in this model.

### *Although no BM-derived insulin-positive cells were detected, the regenerated islets were surrounded by BM-derived CD45-positive cells*

To investigate whether BM-derived cells transdifferentiated into insulin-producing cells in our model, pancreases from STZ+BMT mice on d 35 were immunostained with antiinsulin antibody, followed by an intensive search for both insulin- and GFP-positive cells using confocal fluorescence microscopy. However, no double-positive cells were detected (Fig. 4A), suggesting that regenerated β-cells in STZ+BMT mice are derived from recipient cells. In contrast, intriguingly, GFP-positive, *i.e.* BM-derived, cells were located around islets (Fig. 4A). In STZ+BM-infused mice, no GFP-positive cells were detected around islets (data not shown). Immunostaining with anti-GFP antibody confirmed that GFP-positive cells exist around islets of STZ+BMT mice (Fig. 4B, *black arrows* indicate islets). To identify the lineage of BM-derived cells around islets, we used several antibodies to immunostain lineage markers. GFP-positive cells around islets were CD45-positive (Fig. 4C, *white arrows* indicate islets), although these cells were not positively stained with F4/80, CD68 (macrophage lineage), CD3/CD5 (T cell lineage), or CD20 (B cell lineage) (data not shown), suggesting immature hematopoietic cells. We additionally examined whether these BM-derived cells are positive for an endothelial cell marker, CD31 (PECAM-1). Although a few GFP-positive cells were positive for CD 31 (Fig. 4D, *red arrow*), most BM-derived cells in or around islets were not positively stained with this endothelial marker. Taken together, these observations suggest that donor immature hematopoietic cells, which may be expanded and mobilized to peripheral blood after BMT, initiate β-cell regeneration.

### *Mobilization of BM-derived cells is necessary for the glucose-lowering effect of BMT after STZ administration*

To determine whether BM-derived cell mobilization is pivotal in this process, we investigated the effects of BMT on

**TABLE 1.** Islet numbers and BrdU-positive cells per pancreatic islet cells of STZ-treated mice receiving subsequent BMT

| | Days after BMT | Nos. of islets | Nos. of BrdU-positive cells | Nos. of cells in islets | Percentage of BrdU-positive cells among islet cells |
|---|---|---|---|---|---|
| STZ(−) control | | 94.0 ± 17.3 | 33.3 ± 4.5 | 7174 ± 1487 | 0.51 ± 0.08 |
| | 0 | 62.0 ± 11.9 | 20.6 ± 5.6 | 4658 ± 1019 | 0.47 ± 0.11 |
| | 3 | 48.3 ± 2.0 | 40.7 ± 16.2 | 2974 ± 278 | 1.41 ± 0.51 |
| STZ+BMT (days after BMT) | 7 | 102.7 ± 11.6 | 194.3 ± 33.4[a] | 6159 ± 528 | 3.10 ± 0.31[a] |
| | 10 | 138.0 ± 22.9[a] | 253.0 ± 107.8[a] | 7989 ± 756[a] | 3.40 ± 1.63 |
| | 15 | 113.7 ± 6.9[a] | 64.7 ± 16.3 | 5223 ± 539 | 1.34 ± 0.40 |
| | 25 | 82.0 ± 6.4 | 37.5 ± 0.4 | 3953 ± 157 | 0.95 ± 0.03 |

To calculate numbers of islets and cells per islet and the percentage of BrdU-positive cells among islet cells, we microscopically examined the whole pancreas in 30-μm sections and counted the numbers of islets, islet cells, and BrdU-positive nuclei in islets.

[a] $P < 0.05$ *vs.* d 0 in STZ+BMT group.

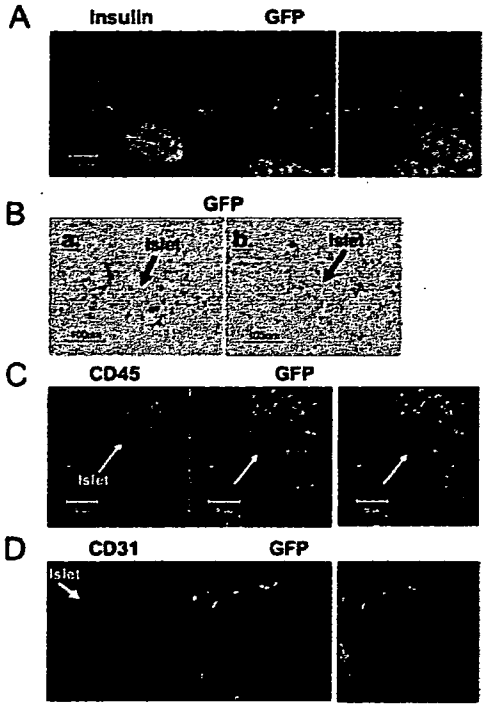


FIG. 4. BM-derived cells in pancreases of STZ-treated mice receiving subsequent BMT. A, BM-derived cells and insulin-positive cells in pancreas of STZ-treated mouse receiving BMT from GFP transgenic mice. Pancreases of STZ-treated mouse receiving subsequent BMT from GFP transgenic mice (35 d after the first STZ). *Left panel*, Insulin-positive cells; *middle panel*, GFP-positive, *i.e.* BM-derived cells; *right panel*, merged image of the *left* and *middle panels*. B, BM-derived cells in pancreases of STZ-treated mice receiving BMT from GFP transgenic mice. *Brown cells* are GFP positive, *i.e.* BM-derived cells, and *arrows* indicate islets. C, CD45-positive and BM-derived cells in pancreas of STZ-treated mice receiving BMT from GFP transgenic mice. *Left panel*, Immunostaining with anti-CD45 antibody. *Red* indicates CD45-positive cells. *Middle panel*, *Green* indicates GFP-positive cells. *Right panel*, Merged image of the *left* and *middle panels*. *Yellow* indicates GFP and CD45 double-positive cells. *Arrows* indicate islets. D, CD31 (PECAM-1)-positive and BM-derived cells in pancreases of STZ-treated mice receiving BMT from GFP transgenic mice. *Left panel*, Immunostaining with anti-CD31 antibody. *Red* indicates CD31-positive cells. *Middle panel*, *Green* indicates GFP-positive cells. *Right panel*, Merged image of *left* and *middle panels*.

β-cell regeneration using eNOS-deficient ($Nos3^{-/-}$) mice as a model for impaired BM-derived cell mobilization. In $Nos3^{-/-}$ mice, mobilizations of hematopoietic stem cells and endothelial progenitor cells from BM were reportedly impaired after myelosuppression. Deficiency in eNOS reportedly reduces hematopoietic recovery in response to 5-fluorouracil treatment due to impaired progenitor cell mobilization (20). Therefore, we performed similar experiments using $Nos3^{-/-}$ mice. First, we compared two BMT groups, *i.e.* $Nos3^{+/+}$ donors to $Nos3^{+/+}$ recipients ($Nos3^{+/+}$ *to* $Nos3^{+/+}$ mice) and $Nos3^{-/-}$ donors to $Nos3^{-/-}$ recipients ($Nos3^{-/-}$ to $Nos3^{-/-}$ mice).

Peripheral white blood cells (WBCs) were counted after lethal irradiation and subsequent BM infusion (Fig. 5A). Myelosuppression after irradiation was profound and recovery of peripheral WBC counts was markedly delayed in $Nos3^{-/-}$ to $Nos3^{-/-}$ mice *vs.* $Nos3^{+/+}$ to $Nos3^{+/+}$ mice. Thus, eNOS deficiency impairs hematopoietic reconstitution after not only 5-fluorouracil treatment but also BMT. We measured the blood glucose levels after STZ administration followed by BMT (Fig. 5B). In $Nos3^{+/+}$ to $Nos3^{+/+}$ mice, STZ-induced hyperglycemia was improved to nearly normoglycemic control levels 40 d after the first STZ administration, consistent with the findings shown in Fig. 1C. In contrast, in $Nos3^{-/-}$ to $Nos3^{-/-}$ mice, BMT did not improve STZ-induced hyperglycemia (Fig. 5B). Thus, eNOS function is essential for improving hyperglycemia after BMT.

In $Nos3^{-/-}$ to $Nos3^{-/-}$ mice, not only mobilization of BMT-derived progenitor cells but also pancreatic endothelial function may be impaired due to systemic eNOS deficiency. Therefore, we performed an additional BMT, *i.e.* $Nos3^{-/-}$ donors to $Nos3^{+/+}$ recipients ($Nos3^{-/-}$ to $Nos3^{+/+}$ mice), whose eNOS is intact in pancreatic blood vessels. In $Nos3^{-/-}$ to $Nos3^{+/+}$ mice, myelosuppression was profound and subsequent recovery of the WBC count was delayed, compared with $Nos3^{+/+}$ to $Nos3^{+/+}$ mice, but this delay in recovery was significantly less severe than that seen in $Nos3^{-/-}$ recipients (Fig. 5A). In $Nos3^{-/-}$ to $Nos3^{+/+}$ mice, blood glucose levels also reached midrange values; the glucose-lowering effects of BMT did occur but were significantly blunted (Fig. 5B). These findings indicate that the lack of hyperglycemia improvement in $Nos3^{-/-}$ to $Nos3^{-/-}$ mice is not attributable solely to the impaired pancreatic endothelial function of recipients. The glucose-lowering effect of BMT inversely correlates with the severity of myelosuppression and delayed recovery, which apparently reflects impaired mobilization of BM cells to peripheral blood.

### *BMT-induced β cell regeneration was impaired in STZ-treated $Nos3^{-/-}$ mice*

To quantify BMT-induced β-cell regeneration in $Nos3^{+/+}$ to $Nos3^{+/+}$ and $Nos3^{-/-}$ to $Nos3^{-/-}$ mice, pancreatic insulin contents 40 d after STZ (30 d after BMT) were measured (Fig. 5C). Compared with $Nos3^{+/+}$ controls without STZ treatment, STZ-treated $Nos3^{+/+}$ mice had markedly lower pancreatic insulin contents. In $Nos3^{+/+}$ to $Nos3^{+/+}$ mice, BMT partially restored pancreatic insulin contents, consistent with our findings that plasma insulin levels were partially restored by BMT (Fig. 1D). In contrast, in $Nos3^{-/-}$ to $Nos3^{-/-}$ mice, BMT effects on pancreatic insulin contents were very limited; pancreatic insulin contents were significantly lower in $Nos3^{-/-}$ to $Nos3^{-/-}$ mice than in $Nos3^{+/+}$ to $Nos3^{+/+}$ mice (Fig. 5C).

Next, changes in islet numbers and percentage of BrdU-positive cells among islet cells in response to BMT were compared between $Nos3^{+/+}$ to $Nos3^{+/+}$ mice and $Nos3^{-/-}$ to $Nos3^{-/-}$ mice. Whereas islet numbers were increased in STZ-treated $Nos3^{+/+}$ to $Nos3^{+/+}$ mice during the period 7–15 d after BMT, islet numbers were significantly less in STZ-treated $Nos3^{-/-}$ to $Nos3^{-/-}$ mice (Fig. 5D). In addition, whereas percentages of BrdU-positive cells among islet cells were markedly increased in STZ-treated $Nos3^{+/+}$ to $Nos3^{+/+}$ mice 7–10 d after BMT, there were significantly fewer such cells in STZ-treated $Nos3^{-/-}$ to $Nos3^{-/-}$ mice (Fig. 5E). These results suggest that impaired BM-derived cell mobilization

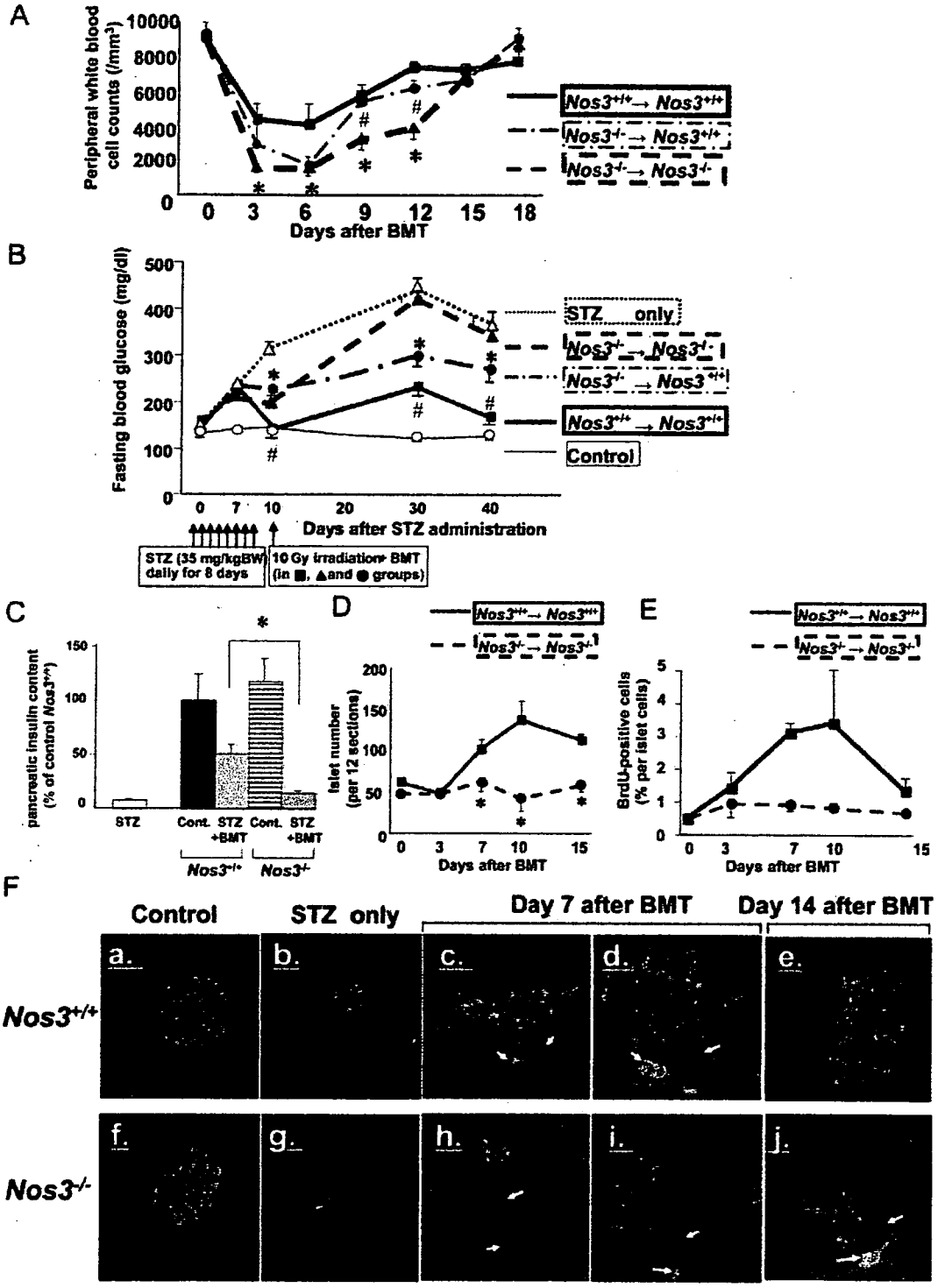


FIG. 5. BMT experiments using *Nos3*$^{+/+}$ and *Nos3*$^{-/-}$ mice. A, Time courses of peripheral WBC counts in *Nos3*$^{+/+}$ and *Nos3*$^{-/-}$ mice receiving BMT. ■, STZ-treated *Nos3*$^{+/+}$ mice receiving BMT from *Nos3*$^{+/+}$ mice; ▲, STZ-treated *Nos3*$^{-/-}$ mice receiving BMT from *Nos3*$^{-/-}$ mice; ●, STZ-treated *Nos3*$^{+/+}$ mice receiving BMT from *Nos3*$^{-/-}$ mice. *, $P < 0.05$ for ▲, compared with ■ group; #, $P < 0.05$ for ●, compared with ▲ group, respectively (n = 5–6 in each group). B, Fasting blood glucose levels of *Nos3*$^{+/+}$ and *Nos3*$^{-/-}$ receiving BMT. ○, Normoglycemic control *Nos3*$^{+/+}$ mice with neither STZ nor BMT; △, STZ-treated *Nos3*$^{+/+}$ mice without BMT (hyperglycemic control); ■, STZ-treated *Nos3*$^{+/+}$ mice receiving BMT from *Nos3*$^{+/+}$ mice; ▲, STZ-treated *Nos3*$^{-/-}$ mice receiving BMT from *Nos3*$^{-/-}$ mice; ●, STZ-treated *Nos3*$^{+/+}$ mice receiving BMT from *Nos3*$^{-/-}$ mice. *, $P < 0.05$ for ●, compared with △ group; # $P < 0.05$ for ■, compared with ● group, respectively (n = 5–6 in each group). C, Pancreatic insulin contents. STZ, STZ-treated *Nos3*$^{+/+}$ mice without BMT; Cont, *Nos3*$^{+/+}$ or *Nos3*$^{-/-}$ mice with neither STZ nor BMT; STZ+BMT, STZ-treated *Nos3*$^{+/+}$ mice receiving BMT from *Nos3*$^{+/+}$ mice and STZ-treated *Nos3*$^{-/-}$ mice receiving BMT from *Nos3*$^{-/-}$ mice. *, $P < 0.05$ between STZ-treated *Nos3*$^{+/+}$ mice receiving BMT from *Nos3*$^{+/+}$ mice and STZ-treated *Nos3*$^{-/-}$ mice receiving BMT from *Nos3*$^{-/-}$mice. D, Time courses of islet numbers after BMT. E, Time courses of BrdU-positive cell percentage per islet cells after BMT. In D and

in *Nos3*$^{-/-}$ mice suppresses BMT-induced β-cell regeneration after acute injury.

*BM-derived CD45-positve cells around islets are important for β-cell regeneration-induced by BMT*

To examine whether impaired mobilization of BM-derived cells in *Nos3*$^{-/-}$ mice affects hematopoietic cell assembly around islets and β-cell regeneration, we compared pancreases from *Nos3*$^{+/+}$ to *Nos3*$^{+/+}$ and *Nos3*$^{-/-}$ to *Nos3*$^{-/-}$ mice using antiinsulin and CD45 antibodies (Fig. 5F). In *Nos3*$^{+/+}$ to *Nos3*$^{+/+}$ mice, substantial numbers of CD45-positive cells were detected in and around the regenerated islets (*red arrows* in Fig. 5F, c–e). No such cells were detected around islets in *Nos3*$^{+/+}$ or *Nos3*$^{-/-}$ mice treated with STZ alone (Fig. 5F, b and g), suggesting that STZ-induced inflammation alone is not responsible for recruiting these cells. In pancreases from *Nos3*$^{+/+}$ to *Nos3*$^{+/+}$ mice, regenerated islets were located near pancreatic ducts and blood vessels (*white arrows* in Fig. 5F, c–e). In contrast, β-cell regeneration was markedly impaired in *Nos3*$^{-/-}$ to *Nos3*$^{-/-}$ mice (Fig. 5F, h–j), and far fewer CD45-positive cells were present in and around islets in *Nos3*$^{-/-}$ to *Nos3*$^{-/-}$ than in *Nos3*$^{+/+}$ to *Nos3*$^{+/+}$ mice. These results support the notion that BMT-induced BM-derived cell mobilization is critical for regeneration of recipient β-cells from stem/progenitor cells in pancreatic ducts.

## Discussion

Recently considerable research attention has focused on pancreatic β-cell regeneration. In particular, several previous studies examined the role of BM-derived cells in β-cell regeneration using BMT (10–18), but no definitive conclusions have yet been reached. In this study, we clearly demonstrate that BMT can regenerate recipient β-cells under certain conditions. Our data supported those of a previous report (14) showing BMT to improve hyperglycemia in STZ-induced diabetic mice via regeneration of recipient pancreatic β-cells. Herein we attempted to elucidate the mechanisms whereby BMT induces β-cell regeneration.

First, we demonstrated that BMT, but not simple BM cell infusion without preirradiation, promotes β-cell regeneration after STZ-induced injury. What are the differences between these procedures? BMT involves lethal irradiation and subsequent BM cell infusion. We confirmed, using FACS analysis, that recipient BM is essentially replaced with that of donor mice after BMT. In contrast, mice receiving BM cell infusion alone without preirradiation showed no BM replacement with donor-derived cells. Myelosuppression and subsequent expansion of donor BM cells take place in BMT. During this process, donor BM cells home to the BM microenvironment and progenitor cells mobilize and expand in the peripheral blood (27). In contrast, simple BM cell infusion does not induce homing or expansion of donor BM cells. Therefore, expansion of immature BM cells, which are rarely detected in peripheral blood in normal circumstances, is likely to be important for β-cell regeneration after BMT. We ruled out the possibility that irradiation suppresses inflammation in response to STZ administration and prevents β-cell injury. STZ-treated mice were exposed to lethal (10 Gy) and sublethal (5 Gy) irradiation without subsequent BM cell infusion. Irradiation alone had no effect on hyperglycemia or β-cell number in STZ-treated mice. Furthermore, in STZ+BMT mice on d 2 after BMT, islet numbers and cell numbers per islet were both significantly decreased by STZ but were restored by d 10. Thus, it is unlikely that irradiation itself protects β-cells.

Next, we found that a major population of post-BMT islets were located near pancreatic ducts and blood vessels. This observation raises possibilities regarding the origins of post-BMT islets. In general, multipotent adult stem cells are located in somatic tissues, which maintain and regenerate impaired tissues (28, 29). However, there is considerable controversy regarding the existence and location of pancreatic tissue stem cells (30, 31). Previous studies have shown pancreatic stem/progenitor cells in ductal epithelium (24–26). However, recent reports suggest that β-cells arise only from self-duplication of preexisting β-cells, *i.e.* β-cells cannot be derived from non-β-cell progenitors (32, 33). In this study, post-BMT islets were located near pancreatic ducts. In addition, BrdU-positive cells were detected in the vicinity of pancreatic ducts in STZ+BMT mice. After islet numbers had been decreased by STZ, a rise above normoglycemic control levels was seen, indicating new islet formation. In addition, BM-derived cells accumulated in and around post BMT-islets. Thus, BM-derived cells are likely to stimulate proliferation and differentiation of pancreatic stem/progenitor cells in ductal epithelium, resulting in new islet formation. Given the observation that BrdU-positive cells in islets expressed insulin, these cells must still have been proliferative after differentiation into pancreatic β-cells. However, further studies, focusing on the origin of newly generated islets, are needed to support this speculation. Whereas BM-derived cells that accumulated around the islets in STZ+BMT mice were CD45 positive, immunohistochemical studies revealed that these cells do not express mature T or B lymphocyte or macrophage markers. Taken together with the finding that simple BM infusion without preirradiation induced neither β-cell regeneration nor accumulation of BM-derived cells (data not shown), we speculate that these immature BM-derived cells send signals triggering proliferation and differentiation of stem/progenitor cells into β-cells. Our next goal is identification of these signals.

To examine the causal relationship between BM-derived cell mobilization and BMT-induced β-cell regeneration, we performed similar experiments using a model of impaired

E, the *thick line* indicates STZ-treated *Nos3*$^{+/+}$ mice receiving BMT from *Nos3*$^{+/+}$ mice, and the *dotted line* STZ-treated *Nos3*$^{-/-}$ mice receiving BMT from *Nos3*$^{-/-}$ mice. *, $P < 0.05$ between STZ-treated *Nos3*$^{+/+}$ mice receiving BMT from *Nos3*$^{+/+}$ mice and STZ-treated *Nos3*$^{-/-}$ mice receiving BMT from *Nos3*$^{-/-}$mice at the same time points. F, Immunostaining of pancreases with antiinsulin and anti-CD45 antibodies. Pancreases from normoglycemic control mice (*Nos3*$^{+/+}$ mouse in a and *Nos3*$^{-/-}$ mouse in f), hyperglycemic control mice (*Nos3*$^{+/+}$ mouse in b and *Nos3*$^{-/-}$ mouse in g), STZ-treated *Nos3*$^{+/+}$ mice receiving BMT from *Nos3*$^{+/+}$ mice (7 d after BMT in c and d, 14 d after BMT in e), and STZ-treated *Nos3*$^{-/-}$ mice receiving BMT from *Nos3*$^{-/-}$ mice (7 d after BMT in h and i, 14 d after BMT in j). *Green* indicates insulin-positive, *red* CD45-positive cells. *Red arrows* indicate CD45-positive cells in and around islets and *white arrows* pancreatic ducts and blood vessels.

BM-derived cell mobilization. Mechanisms underlying mobilization of hematopoietic and endothelial progenitor cells from BM after myelosuppression have been studied in detail (34). After myelosuppression, secreted cytokines/chemokines, such as granulocyte-colony stimulating factor, stromal cell-derived factor, and vascular endothelial growth factor, activate matrix metalloproteinase (MMP)-9 in the BM microenvironment. Activated MMP-9 processes membrane-bound kit-ligand, releases it as soluble kit-ligand (sKitL), followed by binding of sKitL to c-*kit* on the stem cell surface and stimulation of its mobilization from the BM. Because nitric oxide from BM is necessary for MMP-9 activation, sKitL production and the resultant mobilization of BM-derived cells are impaired in *Nos3*$^{-/-}$ mice (20). Therefore, using *Nos3*$^{-/-}$ mice, we examined the effects of BMT on blood glucose levels and glucose β-cell regeneration. We first confirmed that recovery of the WBC count after BMT was significantly delayed in *Nos3*$^{-/-}$ to *Nos3*$^{-/-}$ mice, compared with *Nos3*$^{+/+}$ to *Nos3*$^{+/+}$ mice. Judging from the doubling time of hematopoietic cells, a 1-wk delay in WBC recovery indicates marked impairment of BM cell mobilization by approximately 2 orders of magnitude. In *Nos3*$^{-/-}$ to *Nos3*$^{-/-}$ mice, BMT had virtually no effects on blood glucose levels, pancreatic insulin contents, islet numbers, or percentage of BrdU-positive cells among islet cells. In addition, far fewer CD45-positive cells were detected in and around islets. These results support the notion that BMT-induced BM-derived cell mobilization plays a pivotal role in β-cell regeneration from ductal progenitor cells.

Neovascularization in ischemic regions is also impaired in *Nos3*$^{-/-}$ mice because of decreased mobilization of BM-derived endothelial progenitor cells (20). The microvasculature is well developed in pancreatic islets (35). Endothelial signals are reportedly important for islet development (36), insulin gene expression, and β-cell proliferation (37). In the present study, a small population of BM-derived cells around regenerated islets was positively stained with CD31, although these cells were largely CD31 negative. This observation is consistent with the results of previous report (14). Therefore, in addition to hematopoietic progenitor cells, endothelial progenitor cells mobilized from BM may contribute to β-cell regeneration after BMT by promoting islet microvasculature formation. In addition, BM-derived endothelial progenitor cells have been shown to contribute to neovascularization in impaired tissues, including myocardial (38) and hind limb ischemia (39). Recruitment of these cells reportedly occurs in response to acute injury of β-cells (19). Taken together with the finding that, when BMT was performed 30 d after STZ treatment, hyperglycemia-improving effects were far smaller, acute STZ injury might trigger migration of immature BM-derived cells to the injured pancreas.

We do not rule out the importance of eNOS in pancreatic blood vessels for BMT-induced β-cell regeneration. However, in *Nos3*$^{-/-}$ to *Nos3*$^{+/+}$ mice, which have decreased BM eNOS (because of BM replacement with eNOS-deficient cells) with intact pancreatic eNOS, the blood glucose-lowering effects of BMT were significantly blunted, compared with *Nos3*$^{+/+}$ to *Nos3*$^{+/+}$ mice. Thus, glucose-lowering effects correlated inversely with the severity of myelosuppression and delayed recovery of the peripheral WBC count, suggesting the importance of BM-derived cell mobilization, rather than eNOS activity in pancreatic blood vessels, in BMT-induced β-cell regeneration.

In summary, BMT promotes β-cell regeneration after STZ-induced injury. A series of BMT experiments using *Nos3*$^{-/-}$ mice demonstrated BM-derived cell mobilization to be essential for BMT-induced β-cell regeneration. Acute injury with STZ treatment may trigger recruitment of immature BM-derived cells to the injured pancreas. Recruited BM-derived cells may then stimulate stem/progenitor cells located in the recipient pancreas, resulting in islet regeneration. To our knowledge, this is the first report showing mobilization of BM-derived cells to be involved in β-cell regeneration in diabetic animals. From the viewpoint of clinical application, it is important to study the effects of myelosuppression-inducing reagents, such as antitumor drugs, on pancreatic islet regeneration.

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Adipose Tissue, Inflammation, and Cardiovascular Disease
Adipocyte Signaling and Lipid Homeostasis: Sequelae of Insulin Resistant Adipose Tissue
Diabetic Cardiomyopathy: The Search for a Unifying Hypothesis

Adiposity and Cardiovascular Disorders: Disturbance of the Regulatory System Consisting of Humoral and Neuronal Signals

PPARγ Activation and the Effects on the Vasculature

***Philipp Scherer, Guest Editor***

# Adiposity and Cardiovascular Disorders

## Disturbance of the Regulatory System Consisting of Humoral and Neuronal Signals

Hideki Katagiri, Tetsuya Yamada, Yoshitomo Oka

*Abstract*—Obesity, a major healthcare issue, is associated with significant cardiovascular morbidities, including hypertension and atherosclerosis. Numerous intensive studies conducted this decade have revealed that adipose tissue is a major endocrine organ that secretes a variety of bioactive substances, termed adipocytokines. Adipocytokine secretion profiles are altered as obesity develops, which may increase the risk of obesity-related cardiovascular disorders. For instance, leptin is upregulated in obese subjects and plays important roles in the pathophysiology of obesity-related atherogenesis through multiple mechanisms, such as its proliferative, proinflammatory, prothrombotic, and prooxidant actions. In contrast, adiponectin, which is downregulated in obese subjects, has protective effects against cardiovascular disorders at various atherogenic stages. In addition to these factors secreted by adipose tissue, neuronal circuits involving autonomic nerves are now being recognized as an important metabolic regulatory system and have thus attracted considerable attentions. Alterations in fat accumulation in intraabdominal organs, such as visceral adipose tissue and the liver, send afferent neuronal signals to the brain, leading to modulation of sympathetic tonus and thereby affecting the vasculature. Moreover, these humoral and neuronal signaling pathways communicate with each other, resulting in cooperative metabolic regulation among tissues/organs throughout the body. Further elucidation of these regulatory systems is anticipated to lead to new approaches to devising therapeutic strategies for the metabolic syndrome. (***Circ Res.*** **2007;101:27-39.**)

**Key Words:** adipocytokines ■ autonomic nervous system ■ metabolic syndrome ■ atherosclerosis ■ hypertension

Excess food intake and physical inactivity underlie the growing worldwide epidemic of obesity, not only in the industrialized nations but also in developing countries. A variety of common disorders, eg, hyperglycemia, hyperlipidemia, and hypertension, are common in obese individuals.[1,2] Such disorders are not clustered coincidently, and intraabdominal visceral adiposity has been suggested to play a fundamental role in the simultaneous development of these

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disorders,[3] collectively termed the metabolic syndrome.[4] In addition, one of the major challenges of this syndrome is the high prevalence of cardiovascular diseases arising from atherosclerosis.

Visceral fat accumulation may be directly associated with the development of cardiovascular disease. Epidemiological studies have suggested that visceral adiposity, as evaluated by the waist-to-hip ratio[5] or computed tomography scanning,[6] is related to coronary artery disease independently of body mass index. Recent intensive studies have revealed that humoral factors secreted by adipose tissue contribute to the development of the metabolic syndrome and vascular diseases.

Adipose tissues were long regarded as nothing more than passive fuel storage sites. However, recent studies have revealed that adipocytes, as well as other cells within fat tissues, release numerous biologically active substances, termed adipocytokines, leading to the concept of adipose tissue as a versatile endocrine gland. Obesity, especially visceral fat accumulation, alters adipocytokine secretion profiles, and obesity-related disorders are now recognized as a state of adipose tissue dysfunction. Cardiovascular morbidity in obese individuals might be explained by adipocytokine secretion profile alterations, which result mainly from enlargement of adipocytes and proinflammatory changes in adipose tissue. In addition, recent studies, including ours, have revealed that adiposity in intraabdominal tissues, such as the liver and visceral adipose tissues, directly influences the autonomic nervous system, and thereby modulates sympathetic tonus.

The present review focuses on the effects of different adipocytokines on vascular functions. In addition, we further discuss intertissue communication of metabolic information via the autonomic nervous system in obesity-related disorders.

## Humoral Factors Involved in Metabolic Regulation

### Humoral Factors Derived From Adipose Tissue

Adipocytes produce and secrete a number of bioactive substances, including polypeptides and nonprotein factors that are known to exert a wide variety of effects on glucose and lipid metabolism, energy homeostasis, and cardiovascular function, among others. These substances, collectively called adipocytokines, include leptin, adiponectin, resistin, angiotensinogen, tumor necrosis factor (TNF)-$\alpha$, plasminogen activator inhibitor (PAI)-1, visfatin, retinol-binding protein (RBP)4, fatty acids, sex steroids, and various growth factors. Insulin resistance is an important factor in the development of coronary heart disease, as evidenced by studies in both animal models and humans. Adipocytokines act synergistically or competitively with insulin. Therefore, these factors directly or indirectly affect vascular function and have the potential to provide useful insights into the pathogenesis of vascular disease.

Here we present the current understanding of the complex roles of adipocyte-derived hormones, in particular leptin and adiponectin, in endothelial cell function and the pathogenesis of atherosclerotic vascular disease (Figure 1).

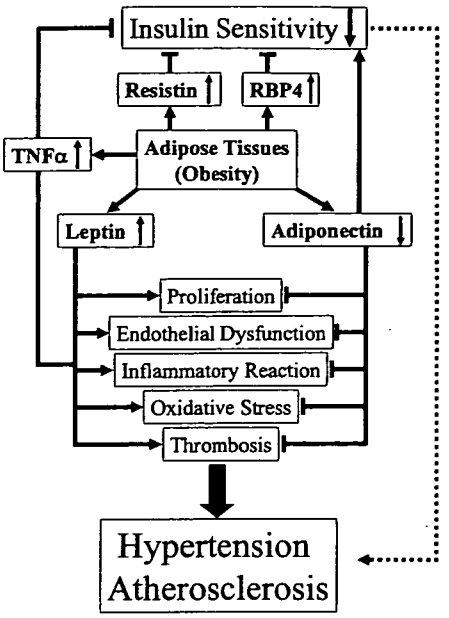


**Figure 1.** Adipocytokines interact in a complex way to regulate vascular function and ultimately the development of cardiovascular diseases.

#### *Leptin*

Leptin was identified by positional cloning in the *ob/ob* mouse model[7] as a key molecule in the regulation of body weight and energy balance. Leptin is a 167-aa secreted protein encoded by the *ob* gene. Leptin is mainly produced and secreted by adipocytes. Leptin acts on the hypothalamus, altering energy intake by decreasing appetite and increasing energy expenditure via sympathetic stimulation of several tissues.[8] Adipocyte leptin expression is transcriptionally regulated, as determined mainly by the status of the energy stores in white adipose tissue and the size of adipocyte sizes. Thus, leptin plays versatile role in maintaining energy homeostasis by communicating information regarding the energy-storage status of adipose tissue to the brain. For instance, with increasing energy storage, the energy balance is negatively regulated by decreased food intake and increased energy expenditure.[9]

Leptin receptors were first isolated from the mouse choroid plexus by expression cloning[10] but are also present in several other tissues, including the hypothalamus. Positional cloning of the *db* locus encoding leptin receptors revealed at least 6 alternatively spliced forms, leptin receptor (Ob-R)a through Ob-Rf. Among these receptor isoforms, Ob-Rb, also termed the long isoform, is highly expressed in the hypothalamus and mediates the anorectic effect of leptin. Ob-Rb contains the longest intracellular domain, which, on ligand binding, activates protein tyrosine kinases of the Janus kinase family–signal transducers and activators of transcription (JAK-STAT) pathway. Other short isoforms, including Ob-Ra, Ob-Rc, Ob-Rd, and Ob-Rf, do not activate the JAK-STAT pathway.[9] Subsequent research demonstrated that the effects of leptin are not restricted to the energy balance. The long form Ob-Rb is expressed throughout the body and has also been detected in endothelial cells.[11] Leptin is a pleiotropic molecule with a wide range of biological actions, including

reproductive functions, regulating the hypothalamic–pituitary–adrenal axis, glucose and insulin metabolism, lipolysis, immune responses, hematopoiesis, and angiogenesis.

*Leptin and the Vasculature*

Several reports have suggested either a vasodilatory or vasoconstrictive action of leptin, which would be direct on the vascular wall. First, the vasodilatory action of leptin is supported by experimental results showing that endothelial-dependent vasorelaxant responses to acetylcholine are markedly impaired in microvessels from leptin-deficient *ob/ob* mice and that leptin restoration reverses the endothelial dysfunction observed in these mice.[12] Leptin has been shown to promote nitric oxide (NO) release from the vascular endothelium, thereby potentially decreasing blood pressure.[13,14] However, in these reports, decreased blood pressure in response to leptin treatment was observed in only sympathectomized rats. In addition, systemic leptin administration does not attenuate the renal and hindlimb vasoconstriction resulting from sympathetic nerve stimulation.[15] These findings suggest that the NO-dependent vasodilatory effects of leptin are insufficient to counter sympathetically mediated vasoconstriction. Furthermore, in vitro treatment of human umbilical vein endothelial cells (HUVECs) with leptin induced endothelin-1, known to be a potent vasoconstrictor.[16] Thus, although high concentrations of leptin may exert vasodilatory effects, the exact vasodilatory actions of leptin remain uncertain.

On the other hand, considerable evidence obtained from animal studies indicates that leptin may modulate arterial pressure through sympathetic mechanisms. In rats, acute intravenous[8] and intracerebroventricular[17] administration of leptin has been shown to increase sympathetic nerve signals to brown adipose tissue, kidneys, adrenal glands, and hindlimbs. Chronic intracarotid[18] and intracerebroventricular[19] administration of leptin also raises blood pressure in rats. Transgenic mice overexpressing leptin in the liver develop hypertension, which is reversed by $\alpha_1$-adrenergic, $\beta$-adrenergic, or ganglionic blockers.[20] Furthermore, despite severe obesity, leptin-deficient *ob/ob* mice have lower blood pressure than lean controls,[21] whereas administering exogenous leptin to *ob/ob* mice raises blood pressure to the levels of lean controls.[20] Thus, leptin has unequivocal sympathoexcitatory actions in rodents. In humans as well, there is a positive relationship between mean blood pressure and serum leptin levels in lean subjects with essential hypertension.[22] In human subjects with widely differing degrees of adiposity, renal norepinephrine spillover correlates with plasma leptin concentrations after adjusting for adiposity,[23] whereas giving leptin to lean subjects for 6 days had no impact on norepinephrine, dopamine, or epinephrine levels in 24-hour urine samples.[24] Further studies are needed to obtain conclusive evidence of the sympathoexcitatory effects of leptin on blood pressure in humans.

*Leptin Resistance and Hypertension*

Obese subjects remain hyperphagic despite their high circulating leptin levels, indicating hypothalamic insensitivity to leptin, a state termed leptin resistance. This was confirmed by clinical trials in which leptin given to obese patients produced only modest effects on body weight.[25] However, despite severe leptin resistance, the sympathoexcitatory effect of leptin, as evaluated by neurography of renal sympathetic nerves, is reportedly preserved after either systemic or central neural administration of leptin.[26]

In mice with dietary obesity, food intake suppression and body weight gain induced by intraperitoneal or intracerebroventricular leptin were significantly attenuated, whereas the renal sympathoexcitatory response to leptin was preserved, leading to substantially elevated arterial pressure. The leptin-dependent increases in arterial pressure were of similar magnitude in mice fed either a high-fat diet or normal chow.[27] These findings led to the notion of selective leptin resistance in which, despite resistance to the anorexigenic effect of leptin, sympathetic nerves are normally activated in response to leptin. In human subjects, there is a strong correlation between leptin plasma concentrations and renal sympathetic activation, as shown in men with widely differing degrees of adiposity.[23] Thus, selective leptin resistance and the resultant sympathetic activation in response to hyperleptinemia may contribute to development of hypertension in patients afflicted with the metabolic syndrome.

*Leptin and Atherosclerosis*

A number of observations indicate a correlation between serum leptin and the pathogenesis of atherosclerotic vascular disease. Human plasma leptin concentrations are independently associated with intima–media thickness in the common carotid artery, an early marker of atherosclerosis.[28] Elevated leptin concentrations in healthy adolescents are associated with decreased arterial distensibility within a broad range of body mass indices.[29] In a major prospective cohort investigation, the West of Scotland Coronary Prevention Study, serum leptin levels were moderately associated with coronary heart disease, independently of other risk factors.[30] In addition, leptin levels independently predict future cardiovascular events in subjects with established coronary atherosclerosis.[31]

In mouse studies as well, there is growing evidence of the contribution of leptin to the development of atherosclerosis. Wild-type mice on an atherogenic diet show leptin elevation and greater neointimal wall thickening after carotid artery injury with high leptin receptor expression in the lesion. In contrast, *ob/ob* mice are markedly resistant to diet-induced formation of atherosclerosis, despite the presence of atherosclerosis risk factors such as diabetes, obesity, and hyperlipidemia. Exogenously administered leptin induces wall thickening in *ob/ob* mice but not in *db/db* mice.[32] Thus, there might be a direct link between hyperleptinemia and an increased risk for cardiovascular disease development in obese subjects. Possible mechanisms underlying the atherogenic actions of leptin will be discussed below.

*Proliferative Actions of Leptin*

The vascular proliferative actions of leptin are exerted mainly via activations of mitogenic factors. For instance, leptin in culture media dose-dependently increases both the migration and the proliferation of rat vascular smooth muscle cells through activation of phosphatidylinositol-3-kinase and mitogen-activated protein kinases.[33] Neointimal formation

after endovascular arterial injury is markedly attenuated in *db/db* mice,[34] suggesting a role for leptin in endothelial intimal layer regeneration after vascular injury. Thus, leptin may contribute to vascular remodeling and perhaps arterial restenosis after angioplasty.

*Proinflammatory Actions of Leptin*

Stimulation of low-grade vascular inflammation is another mechanism whereby leptin may promote both endothelial dysfunction and atherogenesis.[35] In *ob/ob* and *db/db* mice, phagocytosis and the expressions of proinflammatory cytokines, such as TNF-$\alpha$, interleukin (IL)- 6, and IL-12, in macrophages are impaired both in vivo and in vitro. Administering exogenous leptin upregulates both phagocytosis and proinflammatory cytokine production in macrophages collected from *ob/ob*, but not from *db/db*, mice.[36] These observations strongly suggest a physiological role of leptin in modulating inflammatory process.

In a cross-sectional investigation involving healthy young males, leptin was independently associated with C-reactive protein,[37] a widely recognized marker of atherosclerotic vascular risk, although whether this is a causal association is unknown. At present, information regarding the interactions between leptin and various inflammatory reactions in humans is limited, but the proinflammatory actions of leptin are speculated to be involved in vascular remodeling.

*Prothrombotic Actions of Leptin*

Obese subjects appear to be predisposed to thrombosis formation, raising the risk of deep venous thrombosis and pulmonary embolism. Experimental evidence obtained with animal models suggests that leptin might be an important procoagulant factor. Thrombi originating from arterial lesions in *ob/ob* mice are unstable as compared with those in littermate controls. Platelet aggregation is blunted in *ob/ob* and *db/db* mice. Exogenous leptin normalizes thrombus formation and platelet aggregation in *ob/ob*, but not in *db/db*, mice.[38] Bone marrow transplantation from *db/db* to normal mice delays thrombus formation in recipients, suggesting the importance of leptin signaling in platelets in thrombosis formation. Leptin accelerates thrombogenesis by acting on platelets of *ob/ob* mice after vascular injury in vivo.[39] In addition, leptin modestly decreases the expression of thrombomodulin, an antithrombotic protein, in cultured HUVECs.[40] These prothrombotic actions of leptin together might contribute to the elevated risk of developing acute coronary events, venous thrombosis, pulmonary thromboembolism, and thrombotic events after plaque rupture, in obese subjects.

*Prooxidant Actions of Leptin*

Increased oxidative stress has been recognized in experimental animal and human obesity and may contribute pathogenically to the metabolic syndrome.[41]

Numerous reports have shown that leptin increases oxidative stress via multiple mechanisms. In bovine aortic endothelial cells, leptin induces mitochondrial superoxide production by increasing fatty acid oxidation via activation of protein kinase A.[42] In rats, leptin administration for 7 days decreased the activity of paraoxonase-1, an antioxidant enzyme contained in plasma lipoproteins, followed by increased plasma and urinary concentrations of isoprostanes, reflecting increased oxidative stress.[43] By increasing oxidative stress and activating protein kinase C, leptin promotes secretion of atherogenic lipoprotein lipase from macrophages in vitro.[44] Thus, leptin-induced oxidative stress is likely not only to directly damage endothelial and vascular smooth muscle cells but also to increase serum atherogenic factors, contributing to development of atherosclerosis.

Collectively, data from animal and human studies suggest that leptin plays major roles in the pathophysiology of obesity-related atherogenesis by impacting multiple steps, including vascular inflammation, proliferation, calcification, and elevated oxidative stress.

***Adiponectin***

Adiponectin, also termed Acrp30,[45] apM1,[46] AdipoQ,[47] or GBP28,[48] was identified independently by 4 research groups using different approaches, as a protein that is specifically and most abundantly[46] produced in adipose tissue. It has a 20-residue signal sequence, collagen-like motif and globular domain and shows significant homology with collagens X and VIII and complement factor C1q.[49] Adiponectin molecules combine via its collagen domain, producing a wide range of multimer complexes in plasma: a low-molecular-weight trimer, a middle-molecular-weight hexamer, and a high-molecular-weight 12- to 18-mer adiponectin.[50,51]

Plasma adiponectin levels in humans are quite high, normally ranging from 3 to 30 $\mu$g/mL. In contrast to leptin, adiponectin plasma levels correlate negatively with body mass index.[52,53] The negative correlation is stronger between plasma adiponectin levels and visceral adiposity than between this protein levels and subcutaneous adiposity.[54,55] The expression of adiponectin in adipose tissue is reportedly regulated by several mechanisms via humoral and neuronal pathways. As an example, insulin and insulin-like growth factor-1 both upregulate adiponectin expression,[56] whereas TNF-$\alpha$ and activation of the peroxisome proliferators-activated receptor (PPAR)$\alpha$ have the opposite effect.[57] Angiotensin II also reportedly reduces adiponectin production, as described below.[58] In addition, sympathetic activation suppresses adiponectin expression via adrenergic $\beta$ function.[59,60] The mechanism underlying the adiponectin reduction in obese subjects remains unclear, but a plausible explanation is that inflammatory cytokines, eg, TNF-$\alpha$, cause transcriptional suppression and secretory inhibition of adiponectin.[57]

Different types of putative adiponectin receptors have been described. T-cadherin was identified as a receptor for the hexameric and high-molecular-weight species of adiponectin but for neither the trimeric nor the globular species.[61] On the other hand, novel family proteins, designated AdipoR1 and AdipoR2, were found to be receptors for globular and full-length adiponectin.[62] This family of adiponectin receptors is predicted to contain 7-transmembrane domains, despite being structurally and functionally distinct from G protein–coupled receptors. AdipoR1 is abundantly expressed in skeletal muscle, whereas AdipoR2 is expressed mainly in the liver. Very recently, simultaneous disruption of both AdipoR1 and -R2 was reported to abolish adiponectin binding as

well as its actions.[63] The molecular pathways by which adiponectin mediates its effects apparently involve activation of AMP-activated protein kinase (AMPK), PPARα, and p38 mitogen-activated protein kinase signaling pathways,[64] although further investigation is needed in this field.

*Adiponectin and Hypertension*

Lower concentrations of plasma adiponectin have been associated with essential hypertension. Patients with hypertension appear to have significantly lower plasma adiponectin levels than normotensive patients.[65,66] The mechanism underlying this observation may involve the effects of angiotensin II. Infusion of angiotensin II in rats decreased plasma adiponectin levels via signaling through the angiotensin II type 1 receptor.[58] Human subjects with essential hypertension, treated with angiotensin II receptor antagonists or angiotensin-converting enzyme inhibitors, had increased adiponectin concentrations without affecting body mass indices.[67] However, the molecular mechanisms whereby angiotensin II signaling reduces adiponectin production have yet to be clarified.

*Adiponectin and Atherosclerosis*

Lines of evidence obtained from experimental animal models, such as adiponectin overexpression and knockout mice, have indicated protective effects of adiponectin against the development of obesity-related vascular diseases including atherosclerosis.

Adenovirus-mediated overexpression of adiponectin in apolipoprotein E (apoE)-deficient mice attenuates atherosclerotic lesion formation in the aortic sinus as compared with control apoE-deficient mice.[68] Transgenic overexpression of globular adiponectin also ameliorates atherosclerotic lesion formation and diminishes the expression of the class A scavenger receptor in apoE-knockout mice, despite the absence of changes in blood glucose and lipid levels.[69] These effects of adiponectin were confirmed by studies using adiponectin-knockout mice. Adiponectin-knockout mice show increased neointimal hyperplasia and proliferation of smooth muscle cells following acute vascular injury.[70,71] Conversely, adenovirus-mediated reexpression of adiponectin blunts the increase in neointimal thickening observed in adiponectin-knockout mice.[71] These in vivo experiments have demonstrated that adiponectin plays a role in preventing atherosclerotic progression. This conclusion appears to be supported by reports showing that, in humans, mutations and polymorphisms within the adiponectin gene, which are associated with lower adiponectin levels, are associated with coronary artery disease.[72,73]

Adiponectin expression in adipocytes and its plasma levels are upregulated by treatment with thiazolidinediones, agonists for PPARγ.[74] There is mounting evidence that PPARγ agonists reduce the incidence of cardiovascular diseases, including myocardial infarction and stroke, in patients with type 2 diabetes who are at a high risk for macrovascular events.[75] Adiponectin deficiency diminishes the ability of thiazolidinediones to improve glucose tolerance,[76] suggesting involvement of adiponectin in the protective effects of thiazolidinediones against the development of cardiovascular diseases.

*Protective Role of Adiponectin Against Endothelial Dysfunction*

A series of in vitro and in vivo studies has suggested that adiponectin exerts protective actions on endothelial cells, thereby preventing the pathogenic effects of obesity on vascular function.

Adiponectin may exert antiinflammatory properties in part by altering NO levels in the endothelium. In human aortic endothelial cells, adiponectin promotes endothelial NO synthase mRNA and its protein expression, resulting in enhanced NO production via AMPK pathway activation[77,78] Globular adiponectin also reverses oxidized LDL-induced suppression of endothelial NO synthase activity.[78,79] Adiponectin-knockout mice show impaired endothelial-dependent vasodilation when given an atherogenic diet.[66] In addition, adiponectin has antiapoptotic effects on endothelial cells.[80,81] Taken together, these observations indicate that adiponectin protects against endothelial dysfunction through multiple mechanisms.

Adiponectin also inhibits nuclear factor-κB (NF-κB) activation in both endothelial cells and macrophages. Inhibition of endothelial NF-κB signaling by adiponectin treatment suppresses TNF-α–stimulated expression of the proinflammatory cytokine IL-8 as well as adhesion molecules, including intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin, such that the attachment of monocytes to endothelial cells is attenuated.[82,83] Adiponectin-induced suppression of these adhesion molecules was also demonstrated in vivo with adenovirus-mediated overexpression of adiponectin in apoE-deficient mice.[68] In addition, in macrophages as well, adiponectin suppresses NF-κB signaling[84,85] and the expression of class A scavenger receptors, resulting in reduced foam cell formation and the secretion of proinflammatory cytokines.[86] Foam-cell formation is further reduced by adiponectin-induced downregulation of acyl-coenzyme A:cholesterol acyltranferase-1, the enzyme that catalyzes the formation of cholesteryl esters,[87] in macrophages. Adiponectin also enhances expression of the antiinflammatory cytokine IL-10 and the tissue inhibitor of metalloproteinase-1 in macrophages.[88] Through this variety of mechanisms, adiponectin limits the initiation of atherosclerotic plaque formation.

*Protective Role of Adiponectin Against Vascular Remodeling*

The evolution of a fatty streak into a complex lesion is characterized by the proliferation of smooth muscle cells, their migration toward the intima, and their synthesis of collagen. Adiponectin may modulate smooth muscle cell proliferation during the development and progression of vascular lesions. Physiological concentrations of adiponectin significantly suppress both proliferation and migration of human aortic smooth muscle cells in vitro, induced by platelet-derived growth factor-BB, via direct binding with platelet-derived growth factor-BB.[89] Adiponectin was also shown to generally inhibit growth factor-stimulated extracellular signal-regulated kinase signaling. Similarly, adiponectin was found to inhibit smooth muscle cell proliferation through its ability to bind to various growth factors and to interfere

with receptor-mediated cellular responses.[90] As described above, these effects of adiponectin were confirmed by in vivo studies with adiponectin-knockout mice.[70,71] Thus, adiponectin may act as a modulator of vascular remodeling and may favor plaque stabilization via these various mechanisms.

*Protective Role of Adiponectin Against Thrombosis Formation*

Investigations using adiponectin-knockout mice further revealed adiponectin to potentially be an endogenous antithrombotic factor. Compared with wild-type control mice, adiponectin-knockout mice showed enhanced thrombus formation and platelet aggregation at sites of vascular injury, with no differences in either platelet counts or coagulation parameters. Adenovirus-mediated supplementation of adiponectin blunted this enhanced thrombus formation.[91] The antithrombotic actions of adiponectin might well play a protective role against developing acute coronary events and some thrombotic diseases.

*Role of Adiponectin in Protection From Ischemic Heart Disease*

Obesity-related disorders have a major impact on both the incidence and the severity of ischemic heart disease,[92,93] and adiponectin may have a protective function in this setting. Adiponectin treatment inhibits apoptosis of cardiac myocytes and fibroblasts exposed to hypoxia-reoxygenation stress. Blockade of the AMPK pathway by dominant-negative AMPK expression inhibits this adiponectin effect of protecting against apoptosis. In addition, cyclooxygenase-2 is upregulated by adiponectin, leading to increased prostaglandin $E_2$ synthesis. Adiponectin thus appears to protect against myocardial ischemia/reperfusion injury through AMPK-dependent and cyclooxygenase-2–dependent pathways.[94] In adiponectin-knockout mice, larger infarcts are observed after ischemia/reperfusion, which is associated with greater myocardial cell apoptosis and TNF-$\alpha$ expression. Adiponectin replenishment attenuates these damaging effects.[94] Thus, adiponectin may protect myocardial cells from hypoxic stress via both antiapoptotic and antiinflammatory mechanisms. Therefore, adiponectin administration might have a practical clinical application in the treatment of acute myocardial infarction.

### *Other Adipocytokines*

*Tumor Necrosis Factor $\alpha$*

The first clear links among obesity, insulin resistance, and chronic inflammation were provided by a report showing enhanced expression of TNF-$\alpha$, a proinflammatory cytokine, in adipose tissue of obese mice.[95] Lack of TNF-$\alpha$ function improves insulin resistance in obese mice,[96] suggesting an important role for TNF-$\alpha$ in the development of insulin resistance. TNF-$\alpha$ is suggested to be involved in vascular remodeling via proinflammatory and insulin resistant effects. Interestingly, obesity is associated with macrophage accumulation in adipose tissue[97] and TNF-$\alpha$ is apparently derived from infiltrating macrophages,[98] suggesting macrophage infiltration of adipose tissue to play a role in development of obesity-related morbidities.

*Plasminogen Activator Inhibitor-1*

PAI-1 is another adipocytokine, which is highly expressed in adipose tissue and has thrombotic effects.[99] During progressive fat accumulation, PAI-1 expression is markedly enhanced in visceral adipose tissue. Plasma PAI-1 levels correlated significantly with visceral adiposity, as evaluated by computed tomography scanning, in humans.[100] Therefore, PAI-1 secreted from accumulated visceral adipose tissue might play an important role in the development of thrombotic disorders, ie, the ultimate consequences of atherosclerosis.

*Retinol-Binding Protein 4*

In subjects with obesity and type 2 diabetes, GLUT4 glucose transporter expression is selectively decreased in adipocytes.[101] Conversely, adipose-specific GLUT4 disruption secondarily induces insulin resistance in muscle and liver.[102] In this mouse model, RBP4 was identified as an upregulated protein in adipose tissue.[103] Transgenic expression or injections of RBP4 caused insulin resistance in mice, whereas experimentally decreasing RBP4 levels ameliorated insulin resistance in diet-induced obesity. RBP4 enhances hepatic gluconeogenesis and attenuates insulin signaling in skeletal muscle.[103] Serum RBP4 is elevated in insulin-resistant mice and humans with obesity and type 2 diabetes.[104] Thus, RBP4 might play a major role in the development of insulin resistance, although the impact of RBP4 on obesity-related hypertension and vascular diseases remains uncertain.

*Resistin*

Resistin is a member of the newly recognized family of cysteine-rich secretory proteins called resistin-like molecules (RELMs) or FIZZ (found in the inflammatory zone). Resistin is expressed almost exclusively in white adipose tissue and leads to insulin resistance in mice.[105] A few studies focusing on the link between resistin and endothelial functions have recently been published. Resistin promotes endothelin-1 release and also upregulates the expressions of adhesion molecules, monocyte chemoattractant chemokine-1, and pentraxin 3, a marker of NF-$\kappa$B–dependent inflammation, while downregulating the expression of TNF-receptor–associated factor-3, an inhibitor of CD40 ligand signaling in endothelial cells.[106,107] These results suggest that resistin contributes to initiation or perpetuation of the atherosclerotic state. However, unlike murine resistin, human resistin expression is very low in adipocytes while being readily detectable in mononuclear blood cells.[108–110] Therefore, the role of resistin in the development of obesity-related vascular diseases in humans is still uncertain.

## Humoral Factors Derived From the Liver

In addition to adipocytokines, circulating factors secreted by the liver are also involved in systemic metabolic regulation. Members of the angiopoietin-like (Angptl) family of proteins are structurally related to angiopoietins, although their receptors are currently unknown. Angptl3 and Angptl6 (angiopoietin-related growth factor) expressions are restricted mainly to the liver, whereas Angptl4 expression is most abundant in the liver and adipose tissue. Angptl3, -4, and -6

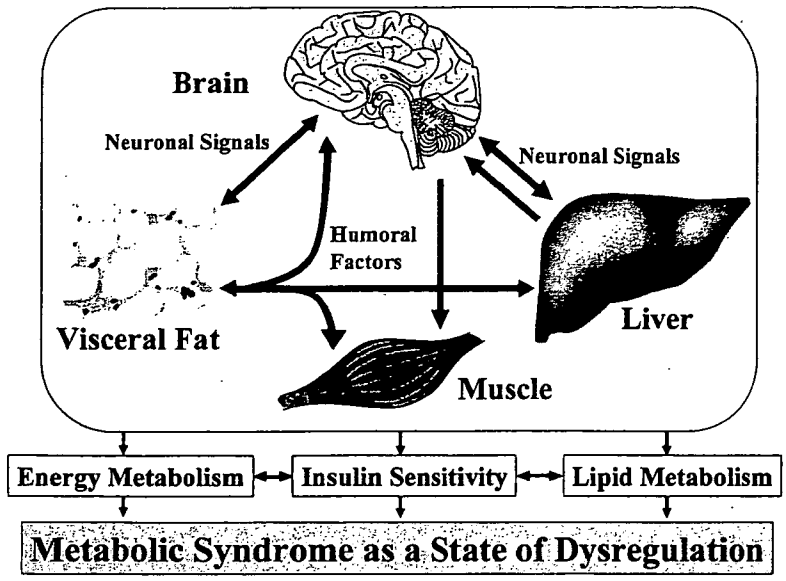


Figure 2. Communications among organs/tissues via humoral and neuronal pathways.

are detected in the systemic circulation, suggesting an endocrine function.

Like the angiopoietins, these Angptl proteins play important roles in angiogenesis, but there are also several reports showing their involvement in triglyceride and energy metabolism as well as insulin sensitivity. Angptl3, a downstream target of the oxysterol receptor liver X receptor,[111] is involved in development of the hypertriglyceridemia.[112] The underlying mechanism appears to be reductions in very-low-density lipoprotein clearance secondary to lipoprotein lipase inhibition[113] and direct activation of lipolysis in adipocytes.[114] In contrast, Angptl6 is suggested to function in counteracting obesity and related insulin resistance through increased energy expenditure.[115]

Angptl4 is also expressed mainly in the liver and adipose tissue, and its expression changes with nutrition status[116] and also according to the activation state of PPARs.[117] Adenovirus-mediated expression of Angptl4 potently decreased blood glucose and improved glucose tolerance, whereas it induced hyperlipidemia, fatty liver, and hepatomegaly. In addition, in patients with type 2 diabetes, serum Angptl4 were lower than in healthy subjects.[118]

Thus, the function, or even dysfunction, of pathways mediated by these humoral factors derived from the liver may contribute to the development of hyperlipidemia and insulin resistance, both major elements of the metabolic syndrome. However, further intensive studies are needed to elucidate the contributions of these factors to cardiovascular disease.

## Neuronal Signals From Intraabdominal Tissues in Response to Metabolic Alterations

In addition to humoral pathways, autonomic nervous system is likely to play an important role in both metabolic and cardiovascular regulation. The central nervous system (CNS) integrates signals from peripheral sites, thereby modulating glucose and energy metabolism as well as blood pressure. At least 2 avenues for these signals, humoral and neuronal, are involved in the underlying mechanisms. Whereas humoral signals including adipocytokines have been intensively investigated in recent years, neuronal signals from adipose tissue and the liver remain largely a mystery. Several recent reports, including ours, have indicated the importance of afferent neuronal signals in response to metabolic alterations, such as adiposity, in intraabdominal organs/tissues. In this regard, afferent signals from intraabdominal organs transmitted by autonomic neurons have attracted considerable attention. Organs/tissues communicate metabolic information each other via humoral and neuronal pathways (Figure 2).

### Neuronal Signals From Adipose Tissues

Fat pads have rich sympathetic fiber innervation. Numerous studies have revealed a role for efferent sympathetic nerves in lipolysis. Various signals from the brain modulate the rate of lipolysis in adipose tissue via sympathetic $\beta$-adrenergic action.[119] In contrast, only a few studies have examined afferent nerve signals from adipose tissue. According to these reports, activation of afferent nerves from intraabdominal (epididymal) adipose tissue results in reflex signals being sent to white adipose tissues via efferent sympathetic nerve activation.[120,121] The functional significance of these afferent signals, however, was not clarified. Research performed by our group has suggested that neural afferent signals from intraabdominal adipose tissue to the brain affect hypothalamic leptin sensitivity, thereby modulating food intake and sympathetic outflow.[122]

Our goal was to determine whether a local reduction in the adiposity of intraabdominal adipose tissue would reverse obesity-related metabolic disorders, in particular, insensitivity to leptin and insulin. Therefore, adenoviral-mediated expression of uncoupling protein (UCP)1, which functions to dissipate energy as heat, was attempted in epididymal adipose tissue of diet-induced obese and diabetic mice in which insulin and leptin resistance had already developed. Despite UCP1 being expressed in epididymal adipose tissue at only very low levels, food intake clearly declined in association with decreased serum leptin levels as well as downregulation of orexigenic neuropeptide Y and upregulation of the anorexigenic precursor neuropeptide proopiomelanocortin in the

hypothalamus. The response to exogenous leptin was enhanced in these mice. In addition, hypophagia could not be duplicated in db/db mice with mutant leptin receptors. Collectively, these findings convincingly demonstrate that very limited UCP1 expression in the intraabdominal fat pad dramatically ameliorates the hypothalamic leptin resistance induced by high-fat-diet feeding. Local dissection of nerves from the epididymal fat pad as well as pharmacological deafferentation abrogated the anorectic effects of adipose UCP1 expression. Taken together, our results suggest afferent nerve signals originating in epididymal fat pads to modulate hypothalamic leptin sensitivity.

Hypothalamic leptin resistance is an important mechanism that maintains the obese state. Therefore, the perturbation of the afferent signals from adipose tissue might contribute to the development of obesity-related disorders, including hypertension and atherosclerosis. Adipose UCP1 expression increases sympathetic outflow, also suggesting the effects of adipose tissue-derived afferent signals on vascular systems. Adipose tissues were long recognized as passive energy storage sites. The discovery of various adipocytokines has raised adipose tissue to the status of a versatile endocrine organ. The aforementioned recent studies may provide additional evidence of the key role of adipose tissue as an important base from which neuronal signals originate. Further elucidation of this new pathway could open a new paradigm enhancing our understanding of adipose functions and dysfunctions, and thereby the pathophysiology of vascular diseases.

## Neuronal Signals From the Liver

Nutrients absorbed from the gut enter the portal vein, a major route to the liver, thereby reaching the liver directly. Thus, given its anatomical location, it seems reasonable for the liver to function as a nutrient sensor and to send signals that regulate systemic metabolism. Signals regarding serum glucose levels from the so-called hepatoportal glucose sensor to the brain have been demonstrated to be carried along afferent vagal nerve pathways.[123] Raising portal vein glucose levels decreases vagal afferent discharges reaching the nuclei of solitary tract neurons, which in turn activates sympathetic efferents to the adrenal glands, liver, splanchnic bed, and pancreas. Because these reflex efferent outputs are all blocked by hepatic vagotomy, it appears that signals triggered by high levels of portal glucose are transmitted through vagal afferents.[123,124] Similarly, hepatic portal infusions of linoleic acid raised hepatic vagal afferent activity, suggesting hepatic vagal afferent involvement in the transmission of signals regarding lipid metabolism to the CNS.[125] In addition, infusion of long-chain fatty acids into the portal vein activates the sympathetic nervous system, thereby elevating blood pressure.[126–128] Therefore, portal nutrient signals may influence systemic blood pressure through afferent vagal and efferent sympathetic nerves. Our recent study provided further evidence of the link between hepatic metabolism and peripheral adiposity[129] through an autonomic nerve circuit consisting of afferent vagal and efferent sympathetic nerve activity.[130]

Hepatic expression of PPARγ, especially PPARγ2, has been shown to be functionally enhanced in a number of obesity models.[131,132] Therefore, to identify the mechanism underlying the interorgan/-tissue communications between the liver and peripheral tissues, including muscle and fat, we overexpressed PPARγ2 in the livers of mice and produced hepatic steatosis using adenoviral gene transfer. Contrary to the increased adiposity in the liver, hepatic PPARγ2 expression markedly reduced adiposity in the periphery with enhanced lipolysis. Systemic metabolic rates were increased, and peripheral insulin sensitivity and glucose tolerance were thus markedly improved. These remote effects were attributed to increased sympathetic outflow into muscle and adipose tissues. Selective hepatic branch vagotomy and pharmacological deafferentation of the vagus completely reversed these remote effects. Thus, hepatic PPARγ2 expression and/or hepatic lipid accumulation stimulates afferent vagal nerve fibers, communicating metabolic information to the brain and producing antiobesity and antiinsulin-resistant effects in muscle and adipose tissue via efferent sympathetic pathways.[130] Fat storage in the liver changes dynamically in accordance with the systemic energy balance and is associated with several features of the metabolic syndrome. Because hepatic PPARγ expression is physiologically associated with obesity, these findings indicate that the liver transmits information regarding excess energy to the CNS via the afferent vagus. When the brain receives this information regarding excess energy storage mediated by leptin from adipose tissues and via the afferent vagus from the liver, the sympathetic nervous system is activated, which in turn enhances energy expenditure and lipolysis, thereby maintaining energy homeostasis. Notably, liver-specific disruption of PPARγ in *ob/ob* mice prevented hepatic steatosis but increased peripheral adiposity, resulting in aggravation of the diabetic phenotype attributable to decreased insulin sensitivity in muscle and fat.[133] Thus, this system consisting of an autonomic nervous circuit appears to function as a protective mechanism against excess calorie intake in physiological settings.

A similar autonomic nerve circuit appears to play an essential role in development of glucocorticoid-induced insulin resistance and hypertension. Glucocorticoid excess is well known to result in insulin resistance and hypertension. In particular, accelerated conversion of glucocorticoid from the inactive to the active form in adipose tissue has phenotypic similarities with the metabolic syndrome.[134] In mice, chronic glucocorticoid exposure leads to insulin resistance and hypertension associated with increased sympathetic tone, renin activity and urinary sodium retention. The underlying mechanism involves hepatic activation of PPARα.[135] Deafferentation, whether surgical or pharmacological, of the hepatic vagus reversed these phenotypic features following chronic glucocorticoid exposure.[136] Taken together, these observations indicate the importance of the vagal afferent pathway in regulating insulin sensitivity and blood pressure. The development of hypertension is attributable to sympathetic activation. Thus, autonomic nerve circuit consisting of hepatic vagal afferent and sympathetic efferent nerves may contribute to the development of obesity-related hypertension. Elucidation of the molecular mechanisms, including the mediators influencing vagal activity, could lead to new therapeutic

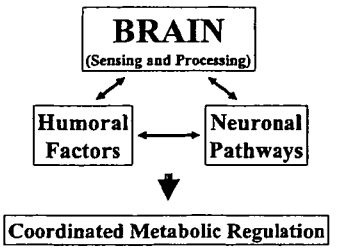


**Figure 3.** The CNS receives peripheral metabolic information and regulates systemic metabolism via humoral factors and neuronal pathways in a coordinated manner.

approaches to the metabolic syndrome and cardiovascular diseases.

## Conclusion

There is a growing body of evidence for a link between obesity and cardiovascular diseases, such as hypertension and atherosclerosis. During this decade, the versatility of adipose tissue as an endocrine organ and as a contributor to disease development has been established. Adipocytokine-mediated crosstalk between adipose tissue and the vascular system is clearly important. In addition, a number of recent studies have shown that tissue-specific knockout mice exhibit unexpected phenotypes, suggesting the presence of currently unknown crosstalk among organs/tissues. Further unraveling the complexities of this interorgan communication would enhance our understanding of the development of obesity-related disorders.

Metabolism is not an independent process, segregated among different organs/tissues, but rather is coordinated and regulated throughout the body. Metabolic regulation coordinated among organs/tissues, which requires communication among these organs/tissues, is apparently essential for maintaining the homeostasis of systemic metabolism, particularly glucose and energy metabolism. Therefore, perturbation of this coordinated control system may lead to the development of metabolic disorders. Recent research advances in this field have revealed myriad complex and important roles of the CNS. The brain receives various forms of metabolic information from peripheral organs/tissues through humoral and neuronal avenues (Figure 3). For instance, leptin acts on the hypothalamus and other brain areas, mediating divergent effects on lipid metabolism and insulin signaling in the brain.[137] Adiponectin also appears to exert central effects on energy metabolism.[138] These inputs are probably integrated and processed in the brain, leading to the transmission of regulatory signals, which in turn induce appropriate systemic responses. In addition, humoral and neuronal signals affect each other, as exemplified by the findings that leptin and adiponectin expressions are regulated by sympathetic activity.[23,60] Further elucidation of these regulatory systems, in much greater detail, may facilitate unraveling the mechanisms underlying metabolic homeostasis and thereby reveal the mechanisms underlying the development of the metabolic syndrome as a state of dysregulation (Figure 2). Moreover, targeting of the coordinated regulatory system consisting of these humoral and neuronal pathways is a potential therapeutic strategy for obesity-related disorders, including cardiovascular diseases.

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## Disclosures

None.

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