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)-α, 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).

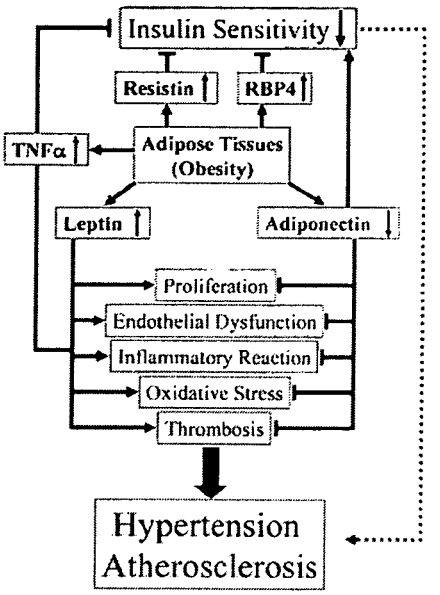

Figure 1. Adipocytokines interact in a complex way to regulate vascular function and ultimately the development of cardiovascular diseases.

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 α_1-adrenergic, β-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-α, 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 μg/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-α and activation of the peroxisome proliferators-activated receptor (PPAR)α 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 β function.[59,60] The mechanism underlying the adiponectin reduction in obese subjects remains unclear, but a plausible explanation is that inflammatory cytokines, eg, TNF-α, 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-α 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 α

The first clear links among obesity, insulin resistance, and chronic inflammation were provided by a report showing enhanced expression of TNF-α, a proinflammatory cytokine, in adipose tissue of obese mice.[95] Lack of TNF-α function improves insulin resistance in obese mice,[96] suggesting an important role for TNF-α in the development of insulin resistance. TNF-α 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-α 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-κB–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

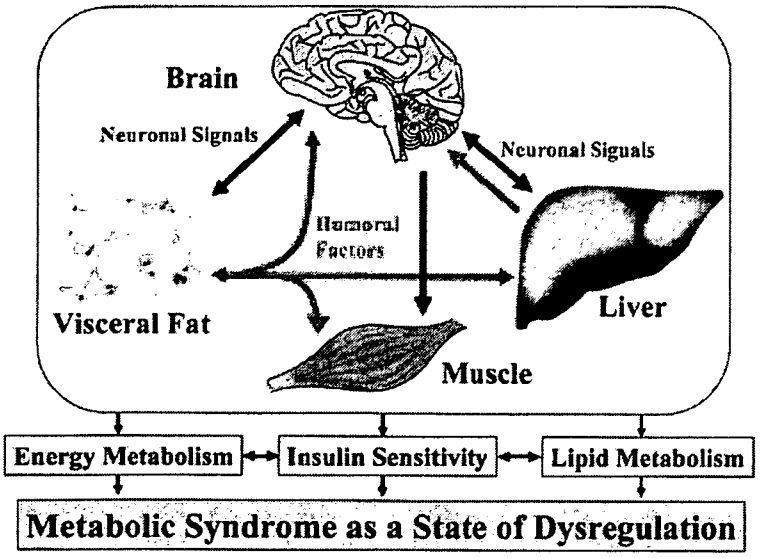

Figure 2. Communications among organs/tissues via humoral and neuronal pathways.

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 β-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

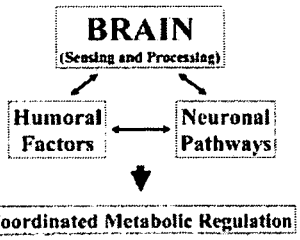

Figure 3. The CNS receives peripheral metabolic information and regulates systemic metabolism via humoral factors and neuronal pathways in a coordinated manner.

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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Increased hepatic expression of ganglioside-specific sialidase, *NEU3*, improves insulin sensitivity and glucose tolerance in mice

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Abstract

Membrane microdomains rich in gangliosides are recognized as being critical for proper compartmentalization of insulin signaling. Plasma membrane–associated sialidase, *NEU3*, is a key enzyme for ganglioside hydrolysis. We previously reported that mice overexpressing *NEU3* mainly in muscles developed severe insulin-resistant diabetes. To examine the possible contributions of *NEU3* to in vivo insulin sensitivity and glucose tolerance, *NEU3* was expressed by using adenoviral vectors in the livers of C57BL/6 mice on standard and high-fat diets, and insulin-resistant KKAy mice on standard diets. Hepatic *NEU3* overexpression paradoxically improved glucose tolerance and insulin sensitivity in the C57BL/6 mice fed standard diets, and glucose tolerance in the C57BL/6 mice fed high-fat diets and in KKAy mice. Hepatic *NEU3* overexpression increased hepatic glycogen deposition and triglyceride accumulation, and enhanced the hepatic peroxisome proliferator–activated receptor γ and fetuin expression in the C57BL/6 mice on standard and high-fat diets, and in KKAy mice. Thin-layer chromatographic analysis demonstrated increased levels of GM1 and markedly reduced GM3 in the livers of mice with hepatic *NEU3* overexpression (*NEU3* mice). Basal and insulin-stimulated tyrosine phosphorylations of insulin receptor substrate 1 were significantly increased, but tyrosine phosphorylations of the insulin receptor and insulin receptor substrate 2 in the *NEU3* liver were unchanged. Insulin-stimulated tyrosine phosphorylations of the insulin receptor were increased in adipose tissues of *NEU3* mice. These results suggest that hepatic *NEU3* overexpression improves insulin sensitivity and glucose tolerance through modification of ganglioside composition and peroxisome proliferator–activated receptor γ signaling. Our findings also provide further evidence that *NEU3* is an important regulator of insulin sensitivity and glucose tolerance.

1. Introduction

Gangliosides are a family of sialic acid–containing glycosphingolipids present on cell surface membranes. Several lines of evidence suggest their functional roles in regulating a wide range of biological processes including cell growth, cell differentiation, and transmembrane signaling [1-4]. Gangliosides are involved in cell-signaling functions as ligands and as modulators of receptor activity [3,5-7]. Sialylparagloboside and the structurally related GM3 ganglioside have been found to inhibit the intrinsic tyrosine kinase activity of soluble insulin receptors [8,9]. Furthermore, GM3 ganglioside depressed insulin-mediated signaling in cultured cells [9,10].

Mice with disrupted *GM3S* gene, which encodes GM3 synthase, such that they lack the capacity to synthesize GM3 ganglioside, had enhanced tyrosine phosphorylation of the skeletal muscle insulin receptor after ligand binding and showed improved responses on glucose and insulin tolerance tests [11]. The *GM3S* knockout (KO) mice were protected from high-fat diet–induced insulin resistance [11]. Insulin resistance with uncoupling insulin receptor activity

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against insulin receptor substrate 1 (IRS-1), which was induced by tumor necrosis factor α in 3T3-L1 adipocytes, was reportedly accompanied by increased GM3 expression caused by elevations of GM3 synthase activity and its messenger RNA (mRNA) [9,12]. When the adipocytes were incubated with exogenous GM3, tyrosine phosphorylation of insulin receptors and IRS-1, as well as glucose uptake in response to insulin stimulation, were suppressed. Obese Zucker fa/fa rats and ob/ob mice, models of insulin resistance, had significantly higher levels of GM3 synthase mRNA in adipose tissues than their lean counterparts [9].

Plasma membrane–associated sialidase, *NEU3*, is a key enzyme for ganglioside hydrolysis [13,14]. Membrane microdomains rich in gangliosides are now recognized as being critical for proper compartmentalization of insulin signaling [4,6,15]. We generated mice overexpressing *NEU3* and reported that these mice developed a diabetic phenotype associated with hyperinsulinemia, islet hyperplasia, and increased beta cell mass [16].

To investigate the role of *NEU3* in insulin and glucose metabolism, we investigated the effects of hepatic *NEU3* overexpression on in vivo glucose tolerance and insulin sensitivity in C57BL/6 mice fed standard and high-fat diets, as well as on KKAy mice receiving a standard diet. Our results show that hepatic overexpression of NEU3 improves insulin sensitivity and glucose tolerance in C57BL/6 mice fed standard diets, as well as glucose tolerance in C57BL/6 mice fed high-fat diets and in KKAy mice. Possible molecular mechanisms underlying improved glucose tolerance and insulin signaling are discussed.

2. Methods

2.1. Preparation of recombinant adenovirus

Adenoviral-mediated gene transfer was used to overexpress the human *NEU3* (*hNEU3*) gene in the livers of male mice. The active adenovirus (AdNEU3) was constructed by using the AdEasy system (Stratagene, La Jolla, CA) and contained the human NEU3 complementary DNA (cDNA) and a short hemagglutinin (HA) tag sequence under the control of cytomegalovirus (CMV) promoters. The adenovirus bearing the bacterial β-galactosidase gene (AdLacZ) was used as a control.

2.2. Animals

Male C57BL/6 mice were housed individually and given free access to a standard diet (65% carbohydrate, 4% fat, and 24% protein) or a high-fat diet (Quick Fat; 60.2% carbohydrate, 15.3% fat, and 24.5% protein; Clea Japan, Tokyo, Japan), starting at 5 weeks of age, for 5 weeks. KKAy mice also were housed individually and given free access to a standard diet. Four weeks after separation, the body weight–matched C57BL/6 mice and KKAy mice received a single dose of AdNEU3 or AdLacZ adenoviruses at a dose of 4×10^8 plaque-forming units by tail-vein injection, resulting in liver-specific infection. Animal study protocols were in accordance with the institutional guidelines for animal experiments at Tohoku University.

2.3. Triglyceride and glycogen contents of the liver

Frozen livers were homogenized, and triglycerides were extracted with $CHCl_3/CH_3OH$ (2:1, vol/vol), dried, and resuspended in 2-propanol [17]. Triglyceride contents were measured using Lipidos liquid (TOYOBO, Osaka, Japan). Glycogen was isolated from 30 to 50 mg of frozen liver by dissolving the tissue in 30% potassium hydroxide saturated with sodium sulfate for 30 minutes at 100°C, followed by ethanol precipitation. Glycogen content was determined by the phenol-sulfuric acid spectrophotometric method at 490 nm [18] and expressed as micrograms of glycogen per milligram of liver.

2.4. Histologic analysis

Livers were removed from the mice on day 7 after adenovirus injection, fixed with 10% formalin, then embedded in paraffin. Tissue sections were stained with periodic acid-Schiff (PAS) and Oil Red O.

2.5. Tyrosine phosphorylation of the insulin receptor, IRS1, and IRS2

Insulin receptor antibody and anti-IRS2 antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Affinity-purified antibody against IRS1 was prepared as described previously [19].

Mice that had been fasted for 16 hours received an injection of 100 μL of normal saline (0.9% NaCl) with or without insulin (standard diets, 2 U/kg body weight; high-fat diets, 10 U/kg body weight), via the tail vein [20]. Liver, epididymal fat tissues and hind-limb muscles were removed 300 seconds later and immediately homogenized. After centrifugation, the resultant supernatants were used for immunoprecipitation with anti-insulin receptor, anti-IRS1, or anti-IRS2 antibody. Immunoprecipitates were boiled in Laemmli buffer containing 10 mmol/L dithiothreitol and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then immunoblotted with antiphosphotyrosine antibody (4G10) as described previously [19]. The immunoblots were visualized with an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK).

2.6. Blood analysis

Blood glucose was assayed with Glucose Ace (Sanwa Kagaku Kenkyusho, Nagoya, Japan). Serum insulin and leptin were determined with enzyme-linked immunosorbent assay (ELISA) kits (Morinaga Institute of Biological Science, Yokohama, Japan). Serum adiponectin concentrations were measured with an ELISA kit (Ohtsuka Pharmaceutical, Tokyo, Japan). Serum total cholesterol, triglyceride, and free fatty acid (FFA) concentrations were determined with Cholescolor liquid, Lipidos liquid (TOYOBO), and NEFA C (Wako Pure Chemical, Osaka, Japan) kits, respectively.

2.7. Glucose and insulin tolerance tests

Glucose tolerance and insulin sensitivity were assessed with intraperitoneal glucose tolerance and insulin tolerance tests. Glucose tolerance tests were performed on fasted (16 hours) mice. Mice were given intraperitoneal glucose (2 g/kg body weight), and blood glucose was assayed immediately before and at 15, 30, 60, and 120 minutes after administration. Intraperitoneal insulin tolerance tests were performed on mice fed ad libitum. Mice received an injection of human regular insulin (0.75 U/kg body weight; Eli Lilly, Kobe, Japan) into the intraperitoneal space, and blood glucose was assayed immediately before and at 20, 40, 60, and 80 minutes after injection.

2.8. Quantitative reverse transcription-polymerase chain reaction–based peroxisome proliferator–activated receptor γ and fetuin gene expression

Total RNA was isolated from 0.1 g of mouse hepatic tissue with ISOGEN (Wako Pure Chemical), and cDNA synthesis was performed with a Cloned AMV First Strand Synthesis Kit (Invitrogen, Rockville, MD) using 5 μg of total RNA. cDNA synthesized from total RNA was evaluated in a real-time polymerase chain reaction quantitative system (Light Cycler Quick System 350S, Roche Diagnostics, Mannheim, Germany).

2.9. Ganglioside-specific sialidase activity assays

Crude homogenates from livers were used for sialidase activity assays with mixed gangliosides (Sigma-Aldrich, St Louis, MO) as substrates in the presence of Triton X-100 as described previously [21,22]. One unit of sialidase was defined as the amount of enzyme catalyzing the release of 1 nmol of sialic acid per hour.

2.10. Glycolipid analysis by thin-layer chromatography

Glycolipids were extracted from mouse tissues as described elsewhere [23,24] and fractionated by thin-layer chromatography (TLC) on high-performance TLC plates (J. T. Baker, Phillipsburg, NJ) in chloroform-methanol-0.5% $CaCl_2$ (60:40:9, vol/vol/vol). Each lane of the plate was loaded with the equivalent of 6 mg, wet weight, of liver tissues, and then was visualized with orcinol-H_2SO_4.

2.11. Statistical Analysis

The statistical significance of differences was assessed by the unpaired Student t test. A P value of $<.05$ was considered significant.

3. Results

3.1. Adenovirus-mediated NEU3 expression in the liver improved glucose tolerance and insulin sensitivity in C57BL/6 mice fed standard diets

The NEU3 adenovirus vector was administered intravenously to C57BL/6 mice on the standard diet (AdNEU3 mice). Mice given the LacZ adenovirus were used as controls (AdLacZ mice). Compared with the controls, the AdNEU3 mice had significantly higher hepatic contents and enzymatic activities of *NEU3* at 7 days after infection (Fig. 1A and B). HA-tagged *NEU3* was not detected in skeletal muscles, adipose tissues, heart, and kidney by Western blotting with anti-HA antibody (data not shown). There were no changes in NEU3 enzymatic activities in skeletal muscle and adipose tissues (data not shown). Thin-layer chromatography demonstrated increases in GM1 and a decrease in GM3 in the livers of AdNEU3 mice compared with those of AdLacZ mice (Fig. 1C).

Food intakes of AdNEU3 mice were significantly lower than those of AdLacZ mice from the 4th through the 7th days after adenovirus injection (Fig. 2A). As a result, the body weights on the 7th and 14th days were significantly decreased in AdNEU3 mice, as compared with AdLacZ mice (Fig. 2B). Blood glucose levels in the ad libitum–fed state were markedly lower in AdNEU3 mice than in control

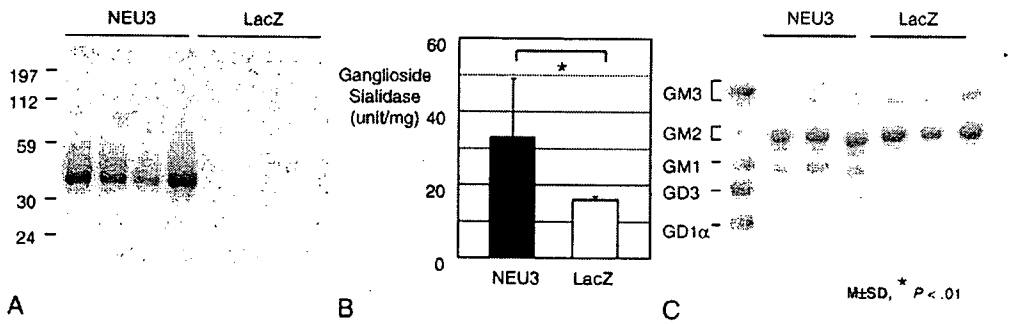

Fig. 1. Hepatic *NEU3* overexpression and ganglioside composition changes in the livers: Western blotting of NEU3 (A), ganglioside-specific sialidase activity (B), and TLC analysis of gangliosides (C). C57BL/6 male mice fed standard diets were injected with 4×10^8 plaque-forming units of adenovirus-containing alpha-galactosidase (AdLacZ) or human NEU3 (AdNEU3) construct via the tail vein. Mice were killed on day 7 after adenoviral injection, and liver samples were collected. A, Western blotting of liver lysates. Liver lysates (45 μg) from AdLacZ or AdNEU3 mice (n = 4) were electrophoresed and immunoblotted with anti-HA antibody (Roche Applied Science, Tokyo, Japan). B, Sialidase activity in liver homogenates was assayed with mixed gangliosides as substrates in the presence of Triton X-100. Similar representative results were obtained from 3 or more experiments, and the data are presented as means ± SD of the 4 mice of each group. *$P < .05$, assessed by unpaired t test. C, Thin-layer chromatography of glycolipids from the livers of AdLacZ and AdNEU3 mice (n = 3). Representative immunoblots and TLC analysis data are presented.

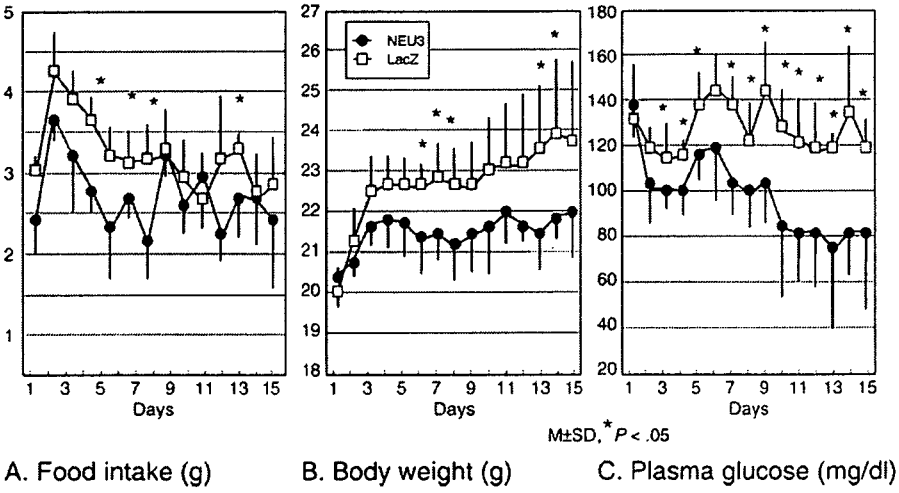

Fig. 2. Hepatic *NEU3* expression reduced food feeding (A), body weights (B), and blood glucose concentrations (C) in C57BL/6 mice fed standard diets. Food feeding (A), body weights (B), and blood glucose levels (C) in the ad libitum–fed state were measured every day after adenoviral administration in AdLacZ (squares) and AdNEU3 mice (circles) for 2 weeks (n = 5 per each group). Similar representative results were obtained from 3 or more experiments, and the data are presented as means ± SD. $*P < .05$, assessed by unpaired t test.

mice from the 3rd to the 15th day after adenovirus injection (Fig. 2C).

On day 4 after adenovirus injection, glucose tolerance tests, ie, intraperitoneal injection of 2 g glucose per kilogram body weight, were performed on C57BL/6 mice that had been fasted overnight. As shown in Fig. 3A, the fasting blood glucose levels did not differ significantly between the AdLacZ and AdNEU3 groups. However, after a glucose load, blood glucose levels were significantly reduced in AdNEU3 mice after 15, 30, 60, and 120 minutes ($P < .05$). Fasting insulin levels did not differ significantly between the AdLacZ and AdNEU3 groups, although plasma insulin levels were significantly reduced in the AdNEU3 mice after 30 minutes ($P < .05$). These results suggest that hepatic *NEU3* expression improved glucose tolerance, possibly through increased insulin sensitivity, after 4 days of administration.

Insulin tolerance tests (0.75 U/kg body weight) were performed in mice fed ad libitum on day 5 after adenovirus injection. The glucose-lowering effect of insulin was significantly improved in the AdNEU3 mice at 20, 40, and 60 minutes after insulin injection, as compared with the AdLacZ mice, further confirming that hepatic *NEU3* overexpression significantly improves insulin sensitivity in C57BL/6 mice fed standard diets.

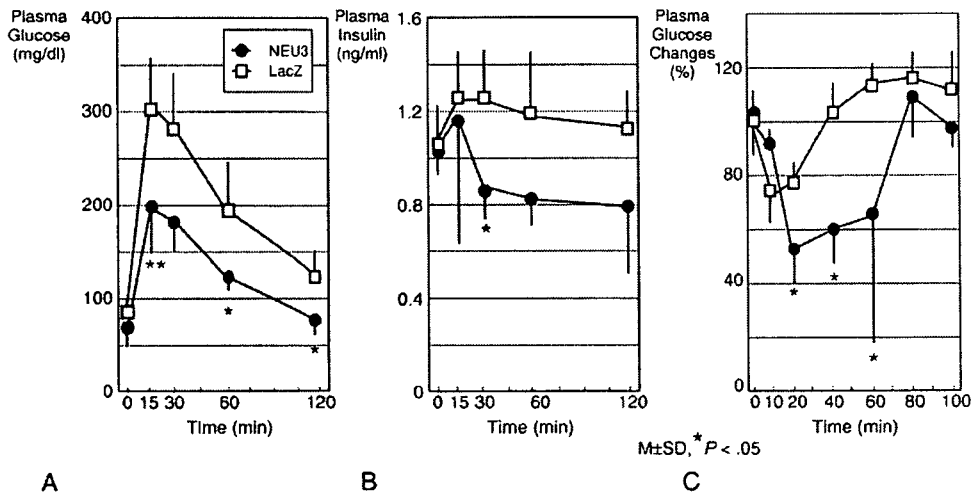

Fig. 3. Hepatic *NEU3* expression improved glucose tolerance and insulin sensitivity in C57BL/6 mice fed standard diets. A and B, Glucose tolerance test. AdLacZ (squares, n = 4) or AdNEU3 mice (circles, n = 4) were fasted 16 hours before intraperitoneal injection with glucose (2 g/kg). Blood glucose levels (A) and insulin levels (B) are determined. C, Insulin tolerance test. AdLacZ (squares, n = 4) and AdNEU3 mice (circles, n = 4) fed ad libitum were intraperitoneally challenged with 0.75 U/kg insulin, and blood glucose levels were determined. Data were expressed as percentages of blood glucose levels immediately before intraperitoneal insulin load. Similar representative results were obtained from 3 or more experiments, and the data are presented as means ± SD. $*P < .05$, assessed by unpaired t test.

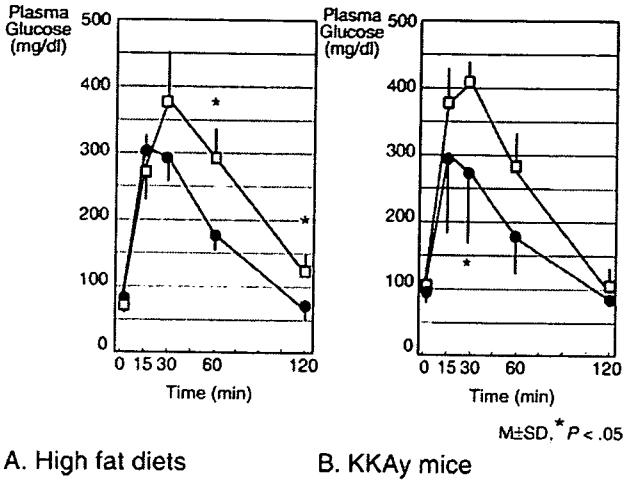

Fig. 4. Hepatic *NEU3* overexpression improved glucose tolerance tests in C57BL/6 mice fed high-fat diets (A) and KKAy mice (B). A and B, glucose tolerance test. AdLacZ (squares, n = 4) or AdNEU3 mice (circles, n = 4) were fasted 16 hours before intraperitoneal injection with glucose (2 g/kg). Blood glucose levels were determined. Significant difference from the AdLacZ value is shown. Data are mean ± SD. $*P < .05$, assessed by unpaired t test.

3.2. *NEU3* overexpression improved glucose tolerance in C57BL/6 mice on high-fat diets and in KKAy mice

When AdNEU3 was administered to the C57BL/6 mice fed high-fat diets, there were no significant differences in food intake or body weight between AdNEU3 and AdLacZ mice (data not shown). However, blood glucose levels in the ad libitum–fed state were markedly decreased in AdNEU3 mice as compared with AdLacZ mice from the 3rd to 15th days after adenovirus injection (data not shown).

Glucose tolerance tests on mice fed high-fat diets demonstrated blood glucose levels to be significantly lower in AdNEU3 than in AdLacZ mice after 60 and 120 minutes (Fig. 4A). AdNEU3 treatment also improved glucose tolerance in KKAy mice (Fig. 4B). These findings indicate that hepatic *NEU3* expression exerted therapeutic effects on diabetic animal models with diet-induced obesity.

3.3. *NEU3* overexpression increased hepatic triglyceride and glycogen accumulation and induced hyperlipidemia

Hepatic overexpression of *NEU3* increased liver weight and hepatic triglyceride deposition in C57BL/6 mice fed standard diets (Fig. 5A and B). AdNEU3 mice had significantly higher hepatic glycogen deposition (Fig. 5C). *NEU3* overexpression also promoted hepatomegaly and triglyceride deposition in the livers of C57BL/6 mice on high-fat diets and of KKAy mice (data not shown). Hepatic *NEU3* overexpression increased plasma triglyceride and total cholesterol concentrations in C57BL/6 mice fed standard diets (Fig. 5D and E). However, there

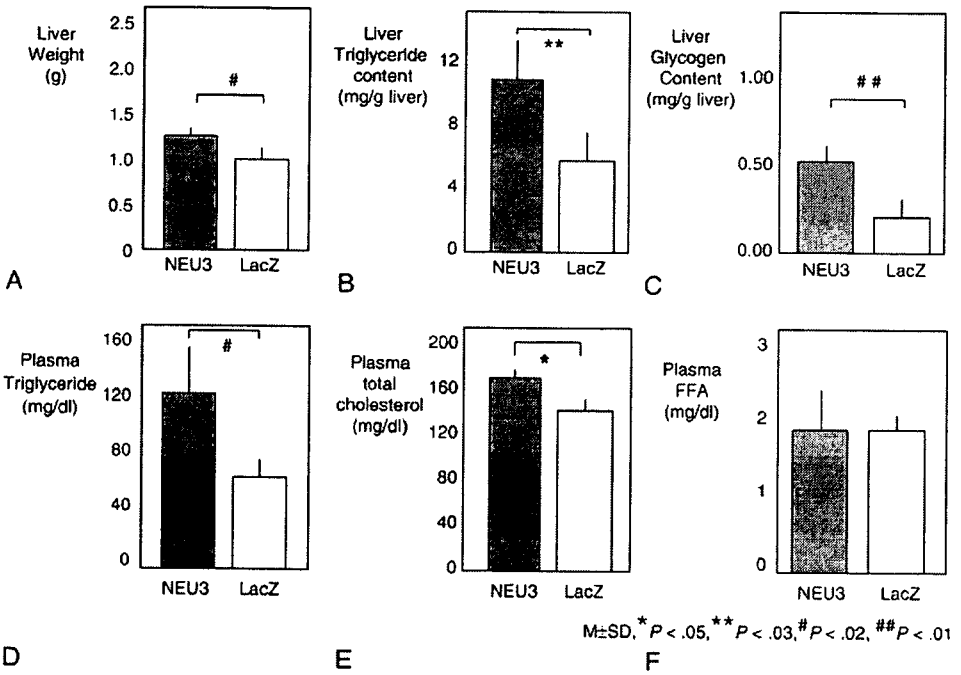

Fig. 5. Hepatic *NEU3* overexpression increased liver weights (A), hepatic contents of triglyceride (B) and glycogen (C), and plasma triglyceride (D) and total cholesterol concentrations (E), but not FFA (F) in C57BL/6 mice fed standard diets. AdLacZ (gray, n = 8) or AdNEU3 mice (white, n = 8) were killed after a 16-h fast on day 7 after adenoviral injection, and livers were removed. Liver weight (A), triglyceride content (B), and glycogen synthesis (C) were determined (n = 8 per group). Blood samples from AdLacZ (gray, n = 8) or AdNEU3 mice (white, n = 8) were collected after a 16-hour fast on day 7 after adenoviral injection. Plasma triglyceride (D), total cholesterol (E), and FFA concentrations (F) were determined. Similar representative results were obtained from 3 or more experiments, and the data are presented as means ± SD. $*P < .05$, $**P < .03$, $^{\#}P < .02$, $^{\#\#}P < .01$, assessed by unpaired t test.

were no significant differences in FFA concentrations between AdNEU3 and AdLacZ mice fed standard diets (Fig. 5F). NEU3 overexpression also increased plasma triglyceride and total cholesterol concentrations, but not plasma FFA in C57BL/6 mice on high-fat diets, and also in KKAy mice (data not shown). There was no difference in serum levels of either adiponectin or leptin between AdNEU3 and AdLacZ mice (data not shown), suggesting that these adipocytokines are not involved in the improvement of glucose tolerance and insulin sensitivity seen in AdNEU3 mice.

These results were confirmed by the histologic findings. PAS staining demonstrated markedly increased glycogen contents in the livers of AdNEU3 mice fed a standard diet compared with AdLacZ mice (Fig. 6A). AdNEU3 mice on standard diets had abundant lipid droplets in their livers compared with AdLacZ mice (Fig. 6B). In the cases of high-fat diet–fed mice (Fig. 6C and D) and KKAy mice (Fig. 6E and F), AdNEU3 mice had abundant glycogen granules and lipid droplets in their livers compared with AdLacZ mice.

3.4. NEU3 overexpression improved insulin-stimulated IRS1 tyrosine phosphorylation in the liver and insulin-stimulated insulin receptor tyrosine phosphorylation in adipose tissues

The results of intraperitoneal glucose tolerance and insulin tolerance tests on days 4 to 5 after adenoviral administration clearly showed that hepatic *NEU3* expression markedly improved glucose tolerance and insulin sensitivity in mice. To explore the potential cellular mechanisms by which hepatic *NEU3* overexpression influences insulin sensitivity and glucose tolerance, we obtained samples of hepatic, skeletal muscle, and adipose tissues after insulin injection on day 4 after adenoviral administration. There was no significant difference in food intake between AdNEU3-and AdLacZ-treated mice until day 4 after adenoviral administration (Fig. 2A).

We first determined whether *NEU3* interferes with activation of upstream insulin signaling components by measuring tyrosine phosphorylation status of the insulin receptors, IRS1 and IRS2. There was no significant

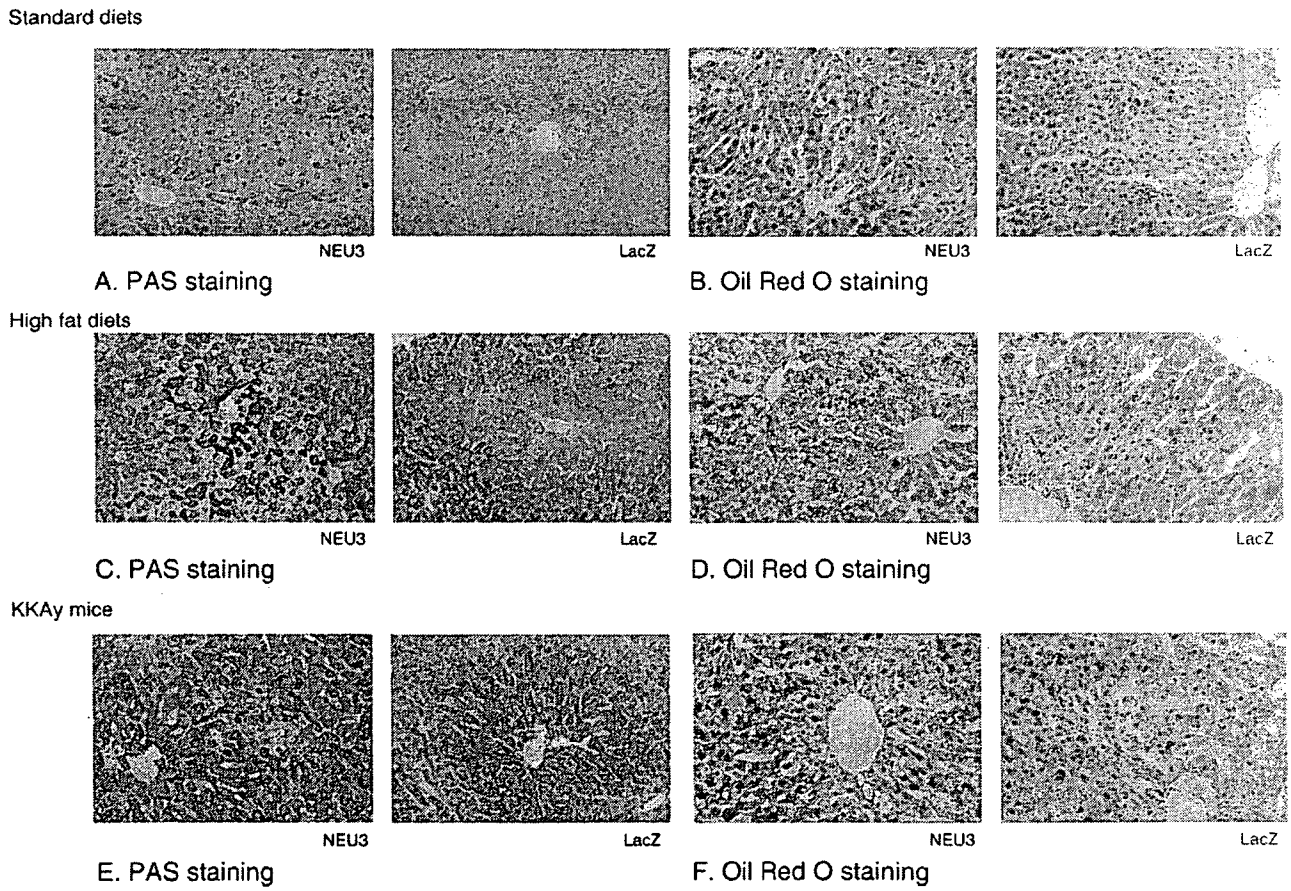

Fig. 6. Hepatic glycogen and fat contents were increased in AdNEU3 mice. Mice were killed after a 16-hour fast on day 7 after adenoviral injection, and livers were removed and subjected to histologic examination. Histologic findings with PAS (A) and Oil Red O staining (B) of the liver from the standard diet C57BL/6 mice treated with AdNEU3 (left) or AdLacZ (right) are shown. PAS (C) and Oil Red O staining (D) of the livers from high-fat diet C57BL/6 mice with AdNEU3 (left) or AdLacZ (right), as well as PAS (E) and Oil Red O staining (F) of the livers from KKAy mice with AdNEU3 (left) or AdLacZ (right) are shown.

difference in insulin-stimulated insulin receptor tyrosine phosphorylation in the liver or skeletal muscles between AdNEU3 and AdLacZ mice (Fig. 7A and B). Basal and insulin-stimulated tyrosine phosphorylations of IRS1 were significantly higher in AdNEU3 than in AdLacZ mice (Fig. 7C). In contrast, IRS2 phosphorylation did not differ between AdNEU3 and AdLacZ mice (Fig. 7D). Basal and insulin-stimulated tyrosine phosphorylation of IRS1 was significantly increased (Fig. 7H), but there were no significant changes in insulin receptor tyrosine phosphorylation in the liver or skeletal muscles of AdNEU3 mice fed high-fat diets (Fig. 7E and F). Insulin-stimulated insulin receptor tyrosine phosphorylation was also increased in adipose tissues of AdNEU3 mice on high-fat diets (Fig. 7G).

Improved insulin sensitivity in the liver was consistent with enhanced basal and insulin-stimulated IRS1 phosphorylation, although insulin receptor phosphorylation was not affected. Improved insulin sensitivity in adipose tissues was also consistent with enhanced insulin receptor phosphorylation in response to insulin administration.

3.5. Hepatic NEU3 expression increased hepatic peroxisome proliferator–activated receptor γ expression in C57BL/6 mice and KKAy mice

As shown in Fig. 8A, insulin-resistant diabetic *NEU3*-transgenic mice [16] had significantly reduced hepatic expression of peroxisome proliferator–activated receptor γ (PPARγ), whereas hepatic overexpression of *NEU3* significantly enhanced hepatic PPARγ expression in mice fed standard and high-fat diets, as well as in KKAy mice. Hepatic fetuin expression was significantly reduced in AdNEU3 mice fed standard diets and in KKAy mice as compared with AdLacZ mice fed standard diets, and KKAy mice (Fig. 8B). The expression level of these genes was consistent with improvement of insulin sensitivity.

4. Discussion

This study demonstrates that *NEU3* overexpression in the liver improves insulin sensitivity and glucose tolerance in C57BL/6 mice fed standard diets. Increased hepatic

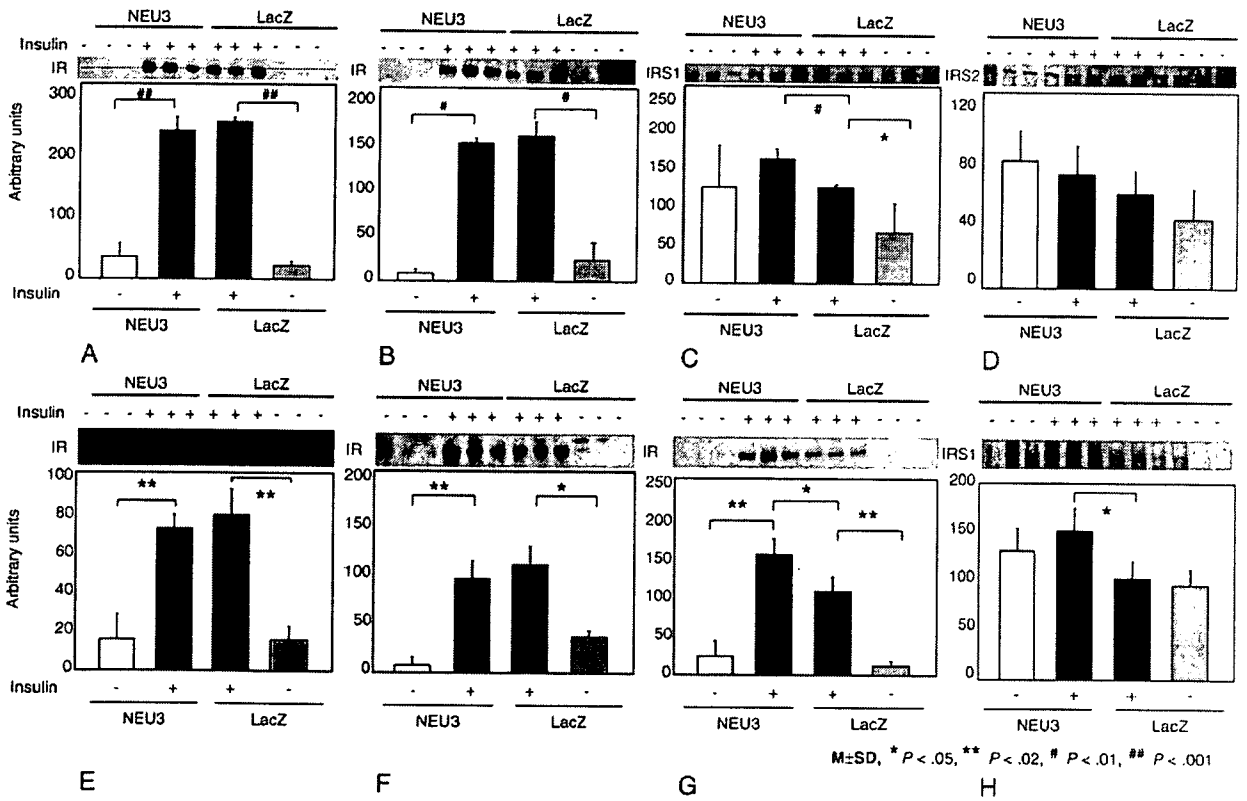

Fig. 7. Insulin-induced tyrosine phosphorylation of the insulin receptor (A, E), IRS1 (C, H), and IRS2 (D) in the livers, and tyrosine phosphorylation of the insulin receptor in skeletal muscles (B, F) and white adipose tissues (G) of C57BL/6 mice on standard (A-D) or high-fat diets (E-H). On day 4 after adenovirus injection, the mice that were fasted for 16 hours received an intravenous injection of 100 μL of saline with or without insulin (standard diets, 2 U/kg body weight; high-fat diets, 10 U/kg body weight). Liver, epididymal fat tissues and hind-limb muscles were removed 5 minutes after the injections. Liver (A, C, D, E, H), adipose tissue (G), and muscle (B, F) lysates from C57BL/6 mice on standard (A-D) or high-fat diets (E-H) were immunoprecipitated with each antibody toward insulin receptor (A, B, E, F, G), IRS1 (C, H), and IRS2 (D) as indicated. Immunoprecipitates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with antiphosphotyrosine antibody (4G10, Upstate). Similar representative results were obtained from 3 or more experiments, and the data are presented as means ± SD. $^{*}P < .05$, $^{**}P < .02$, $^{\#}P < .01$, $^{\#\#}P < .001$, assessed by unpaired t test.

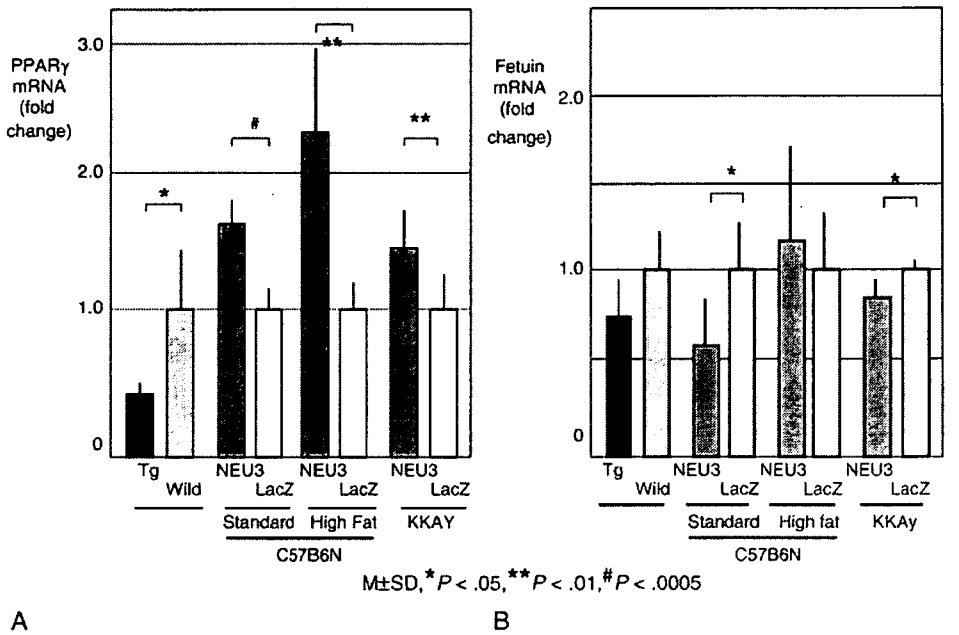

Fig. 8. The gene expression of PPARγ (A) and fetuin (B) in the livers from *NEU3* transgenic and wild C57BL/6 mice and AdNEU3- or AdLacZ-treated C57BL/6 mice on standard or high-fat diets and AdNEU3- or AdLacZ-treated KKAy mice. The relative mRNA levels of PPARγ and fetuin in the livers from *NEU3*-transgenic mice (Tg) and wild C57BL/6 mice [16], and mice treated with AdNEU3 or AdLacZ, as measured by quantitative reverse transcription-polymerase chain reaction. The primers used were the forward primer (5′-GAACGTGAAGCCCATCGAGGAC-3′) and the reverse primer (5′-CTGGAGCACCTTGGCGAACA-3′) for PPARγ, as well as the forward primer (5′-CCCAGTGTCTACTCTGGTGGA-3′) and the reverse primer (5′-CTGTGTTTGGAATAACTTGCAG-3′) for fetuin. The relative amount of mRNA was calculated with β-actin mRNA as the invariant control. Vertical axes represent the fold change in mRNA levels compared with the control mice and those treated with AdLacZ, respectively. The bars represent the fold change of PPARγ and fetuin relative to the mean expression in the controls ± SD (n = 5). Similar representative results were obtained from 3 or more experiments. $^{*}P < .05$, $^{**}P < .01$, $^{\#}P < .0005$, assessed by unpaired *t* test.

contents of glycogen and triglycerides suggest increased hepatic insulin sensitivities in C57BL/6 mice. Hepatic NEU3 overexpression also improves glucose tolerance in 2 types of insulin-resistant mice: C57BL/6 mice on a high-fat diet and KKAy mice.

The present study clearly demonstrates that hepatic NEU3 expression increased tyrosine phosphorylation of IRS1 in the basal and insulin-stimulated states, thereby leading to enhanced insulin sensitivity in vivo. In contrast, *NEU3* overexpression did not affect insulin-stimulated tyrosine phosphorylation of IRS2 or the insulin receptor in the liver. TLC analysis in this study demonstrated increased levels of GM1; in contrast, GM3 was markedly reduced in the livers of AdNEU3 mice. Modification of gangliosides regulates intracellular insulin signaling through direct modulation of insulin receptor tyrosine kinase in the microdomain component of hepatocyte plasma membranes [8-10]. These results suggest that hepatic *NEU3* overexpression improves insulin sensitivity and glucose tolerance through modification of the ganglioside composition, possibly via accelerated degradation of GM3.

This study demonstrates that hepatic *NEU3* overexpression enhances hepatic PPARγ expression, increases triglyceride accumulation, and induces hyperlipidemia. Hyperlipidemia and fatty liver are thought to be part of the metabolic syndrome, and are clinically associated with hyperglycemia, hyperinsulinemia, and insulin resistance [25]. Therefore, our finding that the beneficial effects of hepatic *NEU3* overexpression on glucose homeostasis are associated with hyperlipidemia and fatty liver is somewhat surprising. However, several recent studies on animal models with hepatic modification of Akt, PTEN, PGC-1 and ANGPTL4 suggest that some signaling pathways that reduce blood glucose and improve insulin sensitivity can simultaneously induce hyperlipidemia and fatty liver [20,26-28].

PPARγ expression in the liver is low compared with that in adipose tissues [29]; however, hepatic expression of PPARγ is functionally enhanced in a number of obesity models [30,31]. In addition, liver-specific disruption of PPARγ in obese (ob/ob) mice prevents hepatic steatosis but increases peripheral adiposity and decreases insulin sensitivity in muscle and fat [32]. Synthetic agonists of PPARγ, thiazolidinediones, were shown to reduce hepatic glucose production and increase glycogen synthesis in diabetic animal models [33]. Mice lacking adipose tissue showed increased insulin sensitivity with thiazolidinedione administration, suggesting that thiazolidinediones can enhance insulin sensitivity independently of adipose tissues [30].

We recently reported that adenovirus-mediated expression of PPARγ-2 in the liver induces acute hepatic steatosis,