CO_2. After the C2C12 cells reached subconfluence, differentiation was induced by treatment with DMEM containing 5% horse serum for 7 days, at which time formation of myotubes was maximal. The following chemicals were purchased: H89 from Seikagaku (Tokyo, Japan); NF-κB inhibitor, NF-κB SN50, and BAY11-7082 from Biomol Research Laboratories (Plymouth Meeting, PA); forskolin, TNF-α, palmitate, and iron (II) sulfate heptahydrate from Sigma-Aldrich (St. Louis, MO); human recombinant adiponectin from R&D Systems (Minneapolis, MN); human recombinant globular adiponectin from BioVendor Laboratory Medicine (Modrice, Czech Republic); and 4-hydroxynonenal from Calbiochem (San Diego, CA). Fatty acid solution was prepared by a method described previously (18).

Two-dimensional electrophoresis. A total of 3.0×10^6 murine primary cultured skeletal muscle cells were dissolved in lysis solution (7 mol/l urea, 2 mol/l thiourea, 4% CHAPS, 0.5% IPG buffer, 18 mmol/l dithiothreitol, and 2 mmol/l phenylmethanesulphonylfluoride, pH 8.5). Impurities such as salts, lipids, detergent, and nucleic acids were then removed from samples using a two-dimensional clean-up kit (Amersham Pharmacia Biotech, Amersham, U.K.). Samples were redissolved in rehydration solution and centrifuged at 24,000 rpm for 20 min at 10°C, and insoluble substances were removed. Using 450 μl of solution corresponding to 400 μg of murine primary cultured skeletal muscle cell protein, two-dimensional gel electrophoresis was performed according to the manufacturer's instructions (Amersham Pharmacia Biotech). The gels were Coomassie brilliant blue stained using PhastGel Blue R-350. Colloidal Coomassie blue–stained gels were scanned using a GS-800 calibrated densitometer (Bio-Rad Laboratories, Hercules, CA), and gel images were analyzed using PDQuest 2D-Image-Analysis software (version 7.3; Bio-Rad Laboratories). For this analysis, three independent sets consisting of a control sample gel and an adiponectin-treated sample gel were prepared. For a between-gel comparison, a set of spot-generation conditions was used. To analyze the proteins, we first chose one protein signal to assure that the number of proteins, with signals more intense than that initially chosen, would be ~1,500. Then, we analyzed only these 1,500 protein signals. The computer allowed automatic detection and quantification of protein spots, as well as matching between the control and adiponectin-treated gels. Routine statistical analysis available within the software package was used to identify up- or down-expressed spots. The differentially expressed protein spots were identified by quantitative comparisons with control gels.

Identification of proteins upregulated by adiponectin. Protein spots of interest were excised from the gels and subjected to matrix-assisted laser desorption/ionization–top of flight (MALDI-TOF) mass spectrometry. In-gel digestion of the individual protein spots was done by the following method. Pieces of gel were destained using 200 μl of 50 mmol/l ammonium bicarbonate in 50% acetonitrile, dehydrated in 200 μl of acetonitrile, and then completely dried by vacuuming and centrifuging. The samples were then allowed to expand in digestion buffer containing 100 mmol/l ammonium bicarbonate, 20 μg/ml of trypsin (Promega, Madison, WI), and 0.1% octyl β-D-glucopyranoside (Sigma-Aldrich) at 4°C. After a 30-min incubation, the samples were incubated overnight at 37°C. Peptides were then extracted twice using 0.1% trifluoroacetic acid in 30% acetonitrile with sonication. The peptide solution was vacuum concentrated until it had decreased to 10 μl and desalted according to the manufacturer's protocol. An AXIMA-CFR model MALDI-TOF mass spectrometer (Shimadzu, Kyoto, Japan) was used for mass analysis of tryptic peptide mixtures. Peptides were identified with the Mascot search program (Matrix Science, London, U.K.).

Western blotting and quantitative PCR. Western blotting was performed as previously described (19). Briefly, after incubation with the indicated chemicals, primary cultured skeletal muscle cells were washed with ice-cold PBS, lysed in ice-cold lysis buffer, and then centrifuged at 14,000*g* for 10 min at 4°C. Supernatants including tissue protein extracts were resolved on 10% SDS-PAGE, followed by electrophoretic transfer to a nitrocellulose membrane. Membranes were incubated for 1 h at room temperature with the appropriate primary antibody. Commercial antibodies against phosphor–inhibitor of κB-α (IκB-α), intercellular adhesion molecule (ICAM)-1 FHC FLC, p65 NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA), and IκB-α (Cell Signaling Technology, Palo Alto, CA) were purchased. After blotting with the indicated secondary antibody, detection was performed using an ECL chemiluminescent kit (Amersham Pharmacia Biotech), according to the manufacturer's instructions. Quantitations were performed using a Molecular Imager (Bio-Rad Laboratories). cDNA was synthesized from the purified total RNA using a reverse transcriptase kit (Amersham Pharmacia Biotech), according to the manufacturer's instructions. For quantitative analysis of FHC, manganese superoxide dismutase (MnSOD), and inductible nitric oxide synthase (iNOS), we conducted real-time PCR using an ABI PRISM model 7000 (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The primer sets and probes for murine FHC (assay ID: Mm00850707_g1), murine MnSOD (assay ID: Mm00449726_m1), and murine iNOS (assay ID: Mm00440485_m1) were purchased. Nuclear protein extracts were prepared by separating the cell pellet into two compartments (i.e., the nucleus and the cytosol), as previously described (18).

Small-interfering RNA reagents and transfection. C2C12 myotubes were transfected with small-interfering (siRNA) against FHC (ID 158606, 66915), MnSOD (ID 152022, 71294), and iNOS (ID 156550, 68442) (Applied Biosystems) using the transfection reagent (AM4510; Applied Biosystems), following the manufacturer's protocol. As the control, we utilized the commercially available siRNA control nonsilencing sequence (4611G; Applied Biosystems). The cells were used for experiments 48 h after siRNA transfection.

Generation and transfection of recombinant adenoviruses expressing FHC and adiponectin. A full-length mouse FHC cDNA was isolated from mouse hepatic RNA by reverse-transcriptase PCR. The oligonucleotide sequences used for PCR were as follows: coding strand, 5′-ACCATGACCACCGCGTCTCCCTCGCAAGTG-3′; noncoding strand, 5′-AGCTTAGCTCTCATCACCGTGTCCCAGGGT-3′. The cDNA was subcloned into TA vectors, pCRII (Invitrogen Life Technologies, CA), sequenced to confirm their identities, and were observed to have no unexpected mutations. Adenovirus-expressing recombinant FHC was prepared by homologous recombination of the expression cosmid cassettes containing the corresponding cDNAs and the parental adenovirus genome, as described previously (20). Adenovirus-expressing recombinant adiponectin was prepared as reported previously (21). For adenovirus-mediated transfection, cultured cells were incubated for 2 h in 37°C with DMEM containing the adenovirus-expressing LacZ or FHC, and the growth media were then added. Experiments were performed 3 days after transfection. Mice were treated with recombinant adenovirus, expressing LacZ or adiponectin, by systemic injection into the tail vein.

Assay of intracellular cAMP contents. cAMP was measured in murine primary cultured skeletal muscle cells using a direct enzyme immunoassay kit according to the instructions provided by the manufacturer (Amersham Pharmacia Biotech). Briefly, cell lysates (100 μl) were transferred to a new 96-well microplate coated with donkey anti-rabbit IgG. After addition of 100 μl of rabbit anti-cAMP serum to each well, the microplate contents were gently mixed and incubated at 4°C for 2 h. Then, after addition of 50 μl of cAMP peroxidase conjugate to each well, the microplates were gently agitated and incubated at 4°C for 60 min. We aspirated and washed each well four times with 400 μl of washing buffer and blotted the plate on tissue paper to remove any residual liquid. Next, we immediately dispensed 150 μl of enzyme substrate into each well, followed by mixing on a microplate shaker for exactly 60 min at room temperature. To halt the reaction, we added 100 μl of 1.0 mol/l sulfuric acid to each well. The optical density was determined in a plate reader at 450 nm.

Detection of intracellular ROS production. Intracellular ROS production was monitored by flow cytometry (Becton Dickinson, Franklin Lakes, NJ) using 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA). Cells were stimulated with the indicated reagents in culture dishes and incubated for 24 h. Then, these cells were washed twice in PBS, followed by addition of 10 μmol/l CM-H_2DCFDA in PBS, and finally placed in the dark at 37°C for 1 h. The cells were washed once, harvested, and suspended in 500 μl PBS. Dead cells were excluded by adding 10 μmol/l propidium iodide, a nuclear stain to which viable cells are impermeable. ROS levels were measured by flow cytometrically by determining the mean fluorescent intensity relative to that of the control group. Using this method, we were able to measure not only H_2O_2 but also hydroxy radical (·OH) or peroxynitrite ($ONOO^-$). As it was important to measure hydroxy radicals (·OH), generated by the Fenton reaction, we adopted this method.

Animals. Nine-week-old male mice (C57BL/KsJ, $n = 14$) were purchased from Clea Japan (Osaka, Japan). After a 2- to 3-day acclimatization period, all mice were maintained on a 12:12-h light-dark cycle, fed a standard rodent diet ad libitum, and given unlimited access to water. The mice were divided into a LacZ-transferred group (control construct) and an adiponectin-transferred group (adiponectin construct), and adenovirus-mediated gene transfer was performed. Before they were killed, the animals were fasted for 8 h. Three days after virus infection, increased serum adiponectin levels were confirmed using both a mouse/rat adiponectin ELISA kit (Otsuka, Tokushima, Japan) and immunoblot analysis with anti-murine adiponectin antibody (Chemicon International, Temecula, CA). Then, total hind limbs were removed and immediately homogenized with a Polytron homogenizer in six volumes of solubilization buffer. Extracts were centrifuged at 15,000*g* for 30 min at 4°C, and the supernatants were used as samples for immunoblotting with anti-FHC antibody.

Statistical analysis. All data were expressed as means ± SE. The statistical significance of differences between groups was assessed with the unpaired Student's *t* test using Stat View software (version 5.01; SAS Institute, Cary, NC). A P value <0.05 was considered statistically significant.

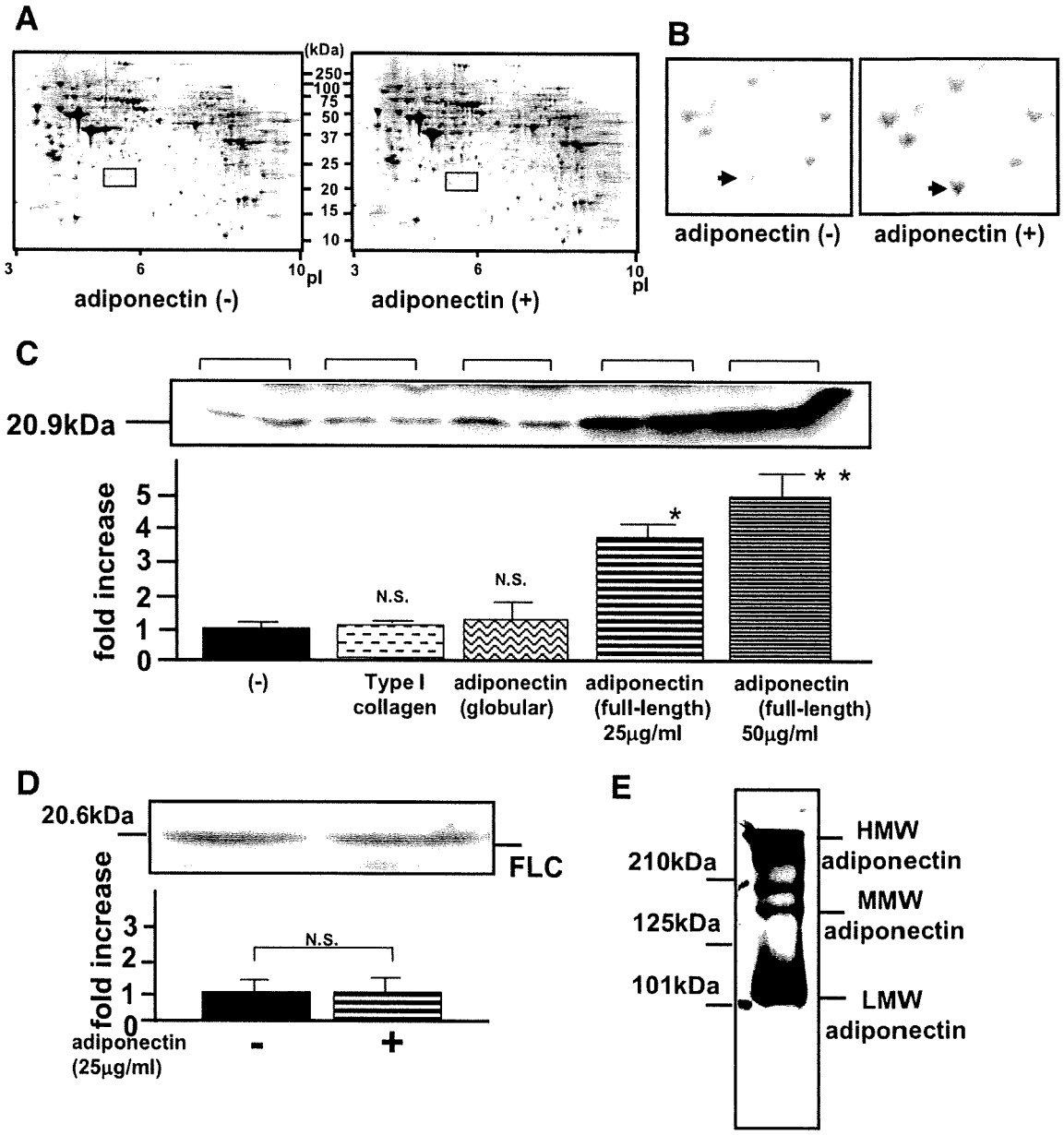

FIG. 1. Effects of incubation with HMW adiponectin on primary cultured skeletal muscles. Primary cultured murine skeletal muscles were incubated for 12 h in the presence (+) or absence (−) of adiponectin (25 μg/ml). *A*: Whole images of Coomassie brilliant blue–stained gels for two-dimensional electrophoresis in the absence (*left*) or presence (*right*) of adiponectin. *B*: Magnified images of two-dimensional electrophoresis revealing a spot apparently altered by adiponectin treatment (arrows). Three two-dimensional electrophoresis sets yielded similar results. *C–E*: After incubation with the indicated ligands, primary cultured skeletal muscle cells were lysed in ice-cold lysis buffer and centrifuged at 14,000*g* for 10 min at 4°C. Supernatants including tissue protein extracts were resolved on 10% SDS-PAGE, followed by electrophoretic transfer to a nitrocellulose membrane. Membranes were incubated for 1 h at room temperature with antibody against mouse FHC (*C*) or FLC (*D*). *E*: The recombinant full-length human adiponectin, which was expressed in a mouse myeloma cell line NS0, was resolved on 7.5% SDS-PAGE under nonreducing conditions and investigated by immunoblotting with anti-adiponectin antibody. After blotting with the indicated secondary antibody, detection was performed using an electrochemiluminescence chemiluminescent kit according to the manufacturer's instructions. Representative data from four independent experiments are presented. *Significant difference ($P < 0.05$) relative to FHC expression in control cells. **Significant difference ($P < 0.05$) relative to FHC expression with 25 μg/ml of adiponectin. N.S., not significant relative to control cells in the absence of adiponectin.

RESULTS

Identification of proteins upregulated by adiponectin. We treated murine primary cultured skeletal muscle cells with recombinant full-length human adiponectin, which was expressed in the mouse myeloma cell line NS0 and purified, and then we searched, using two-dimensional electrophoresis, for proteins upregulated more than three-fold by adiponectin as compared with untreated cells. As confirmed by immunoblotting under nonreducing conditions (Fig. 1*E*), the adiponectin species used in this experiment may be atypical because these were essentially mixtures of the HMW and LMW isoforms of adiponectin, with little of the MMW form. The gels were stained with Coomassie brilliant blue (Fig. 1*A*) and scanned using a GS-800 calibrated densitometer, and gel images were analyzed. We selected 1,500 protein signals. Among these, only one protein was increased (6.3-fold) with adiponectin incubation. The protein spots in the

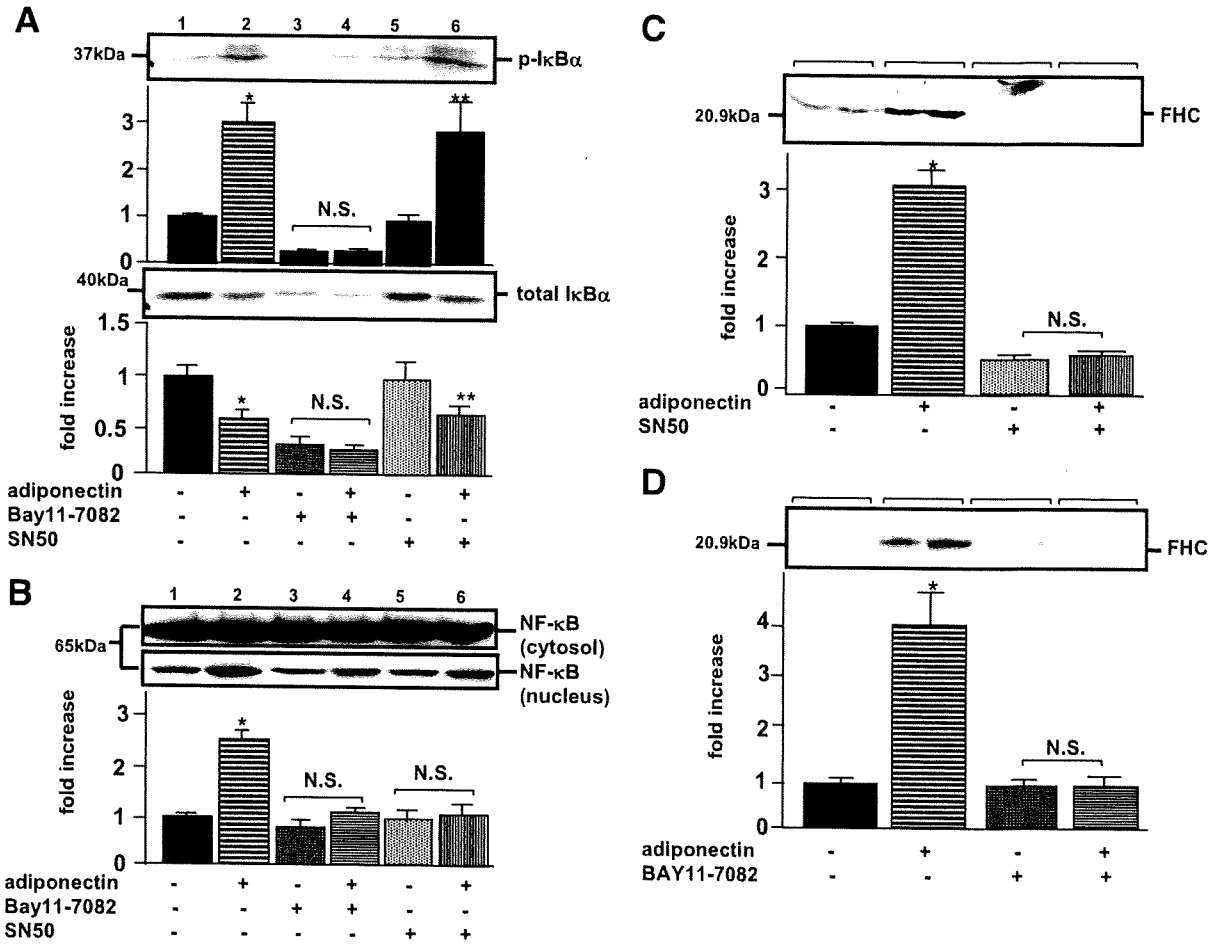

FIG. 2. Effects of IκB/NF-κB inhibitor on FHC in murine primary cultured skeletal muscle cells. Primary cultured skeletal muscle cells were pretreated with 100 μmol/l BAY11-7082 or 50 μg/ml NF-κB SN50 at 1 h before the cells were incubated with 40 μg/ml of adiponectin for 12 h. Cells were lysed in ice-cold lysis buffer and centrifuged at 14,000*g* for 10 min at 4°C. Supernatants including tissue protein extracts were subjected to SDS-PAGE. Transferred membranes were incubated for 1 h at room temperature with antibody against phosphor-IκB-α (*A*, *upper panel*), IκB-α (*A*, *lower panel*), p65 NF-κB (*B*), and FHC (*C* and *D*). After blotting with the indicated secondary antibody, detection was performed using an electrochemiluminescence chemiluminescent kit. Representative data (one sample each for *A* and *B*; two each for *C* and *D*) from four independent experiments (two samples for each experiment) are presented. Values are means ± SE. *Significant difference ($P < 0.05$) relative to IκB-α phosphorylation (*A*, *upper panel*), total IκB-α (*A*, *lower panel*), p65 NF-κB (*B*), or FHC expression (*C* and *D*) of control cells in the absence of adiponectin. **Significant difference ($P < 0.05$) relative to IκB-α phosphorylation (*A*, *upper panel*) or total IκB-α (*A*, *lower panel*) of paired control cells in the absence of adiponectin (*lane 5*). N.S., not significant relative to IκB-α phosphorylation (*A*, *upper panel*), total IκB-α (*A*, *lower panel*), p65 NF-κB (*B*), or FHC expression (*C* and *D*) of paired control cells in the absence of adiponectin.

absence or presence of adiponectin are indicated by the arrows in the magnified figure (Fig. 1*B*). We excised the protein spot from the gel (Fig. 1*B*, *right panel*) and identified four peptides, which were matched to FHC sequences (a.a.54–63, a.a.109–143, a.a.147–156, and a.a.158–172) using MALDI-TOF-MS analysis. The Score and Expect of the Mascot Search were 76 and 0.0022, respectively, both of which are highly definitive for FHC. As shown in Fig. 1*C*, FHC protein expressions were significantly increased in an adiponectin concentration–dependent manner but were unaffected by incubation with the same concentration of type I collagen or recombinant globular adiponectin, suggesting FHC upregulation to be specific to the multimer formation of adiponectin. Next, we assessed whether the expression of FLC is also upregulated by adiponectin. However, FLC expression was not altered (Fig. 1*D*).

NF-κB activation is involved in FHC upregulation by adiponectin. As FHC was reported to be transcriptionally upregulated in response to NF-κB activation (22), we next investigated whether adiponectin enhances NF-κB activity in primary skeletal muscle cells. The NF-κB bound to IκB-α is generally located in the cytosol before activation. In response to stimuli, IκB-α proteins are degraded, a process controlled by IκB-α phosphorylation, resulting in nuclear translocation of NF-κB and subsequent activation of NF-κB target gene transcription. When the cells were incubated with adiponectin, phosphorylation of IκB-α (Fig. 2*A*, *upper panel*, *lanes 1 and 2*) and a decrease in total IκB-α (Fig. 2*A*, *lower panel*, *lanes 1 and 2*) were observed, revealing that adiponectin actually stimulates NF-κB activation. The IκB-α phosphorylation required at least 3 h of incubation, suggesting that secondary effects might be involved in adiponectin-induced IκB-α phosphorylation. NF-κB SN50, an inhibitor of NF-κB translocation into the nucleus, did not affect the Iκ-B phosphorylation by adiponectin (Fig. 2*A*, *lanes 5 and 6*), whereas it was abolished by incubation with BAY11-7082, an inhibitor of IκB-α phosphorylation (Fig. 2*A*, *lanes 3 and 4*). To confirm that SN50 inhibits NF-κB translocation to the nucleus, we separated the myocyte-lysates into two compartments (i.e., nuclear extracts and cytosol) and performed immu-

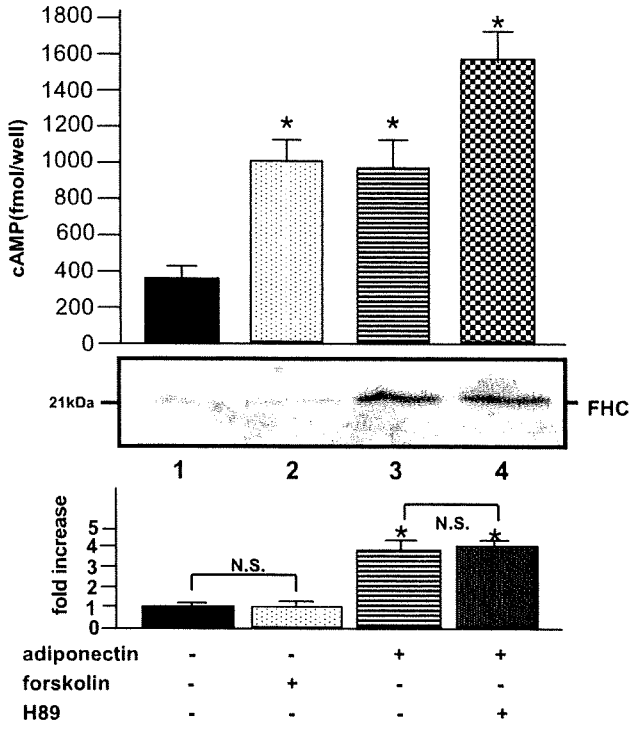

FIG. 3. Effects of recombinant human adiponectin on cAMP content of murine primary skeletal muscle cells. Primary skeletal muscle cells, cultured in 96-well plates, were exposed to 50 μg/ml of adiponectin and 5 μmol/l forskolin for 12 h. Adiponectin-treated cells were also pretreated with 1 μmol/l H89, a PKA inhibitor, for 1 h before the addition of adiponectin. The cAMP assay was performed according to the manufacturer's instructions. Cell lysates from primary cultured skeletal muscle cells were subjected to SDS-PAGE, followed by electrophoretic transfer to a nitrocellulose membrane. Membranes were incubated for 1 h at room temperature with antibody against mouse FHC. Detection was performed using an electrochemiluminescence chemiluminescent kit according to the manufacturer's instructions. Representative results from four independent experiments are presented. Values are means ± SE. *Significant difference ($P < 0.05$) relative to control cells in the absence of ligands (*lane 1*). N.S.; not significant relative to paired control cells in the absence of forskolin (*lane 1*) or H89 (*lane 3*).

noblotting using the anti-p65 NF-κB antibody. As shown in Fig. 2*B*, increases in nuclear NF-κB proteins were observed with adiponectin incubation (Fig. 2*B*, *lane 2*), indicating translocation of the activated NF-κB into the nucleus, whereas no significant translocation of NF-κB proteins was observed when both adiponectin and SN50 were present (Fig. 2*B*, *lane 6*). In addition, FHC upregulation by adiponectin was completely abolished by NF-κB SN50 (Fig. 2*C*) or BAY11-7082 (Fig. 2*D*), indicating that FHC was upregulated by adiponectin via an NF-κB–dependent pathway. Taking into consideration the reported upregulation of FHC by cAMP via a proximal *cis*-acting element containing the CCAAT motif (23), we further examined whether FHC is regulated by a cAMP–protein kinase A (PKA)-dependent pathway. When incubated with adiponectin, cAMP levels inside primary cultured muscle cells were increased by 2.8-fold with FHC upregulation (Fig. 3, *lane 2*). Unexpectedly, H89, a PKA inhibitor, failed to block this FHC upregulation by adiponectin (Fig. 3, *lane 4*). Furthermore, forskolin increased cAMP levels in these cells without FHC upregulation (Fig. 3, *lane 2*). These results suggest that cAMP elevation in response to adiponectin treatment was not associated with FHC upregulation.

The effects of adiponectin, TNF-α, and free fatty acids on NF-κB target gene in HUVECs. To investigate whether adiponectin increases FHC in other types of cultured cells, we further examined the NF-κB activation of HUVECs. When HUVECs were treated with adiponectin, ICAM-1, which is primarily regulated by the NF-κB transcription factor, was slightly, but significantly, increased (Fig. 4*A*). In contrast, TNF-α markedly increased ICAM-1 expression (Fig. 4*B*). These ICAM-1 inductions by adiponectin or TNF-α were both inhibited by the addition of BAY 11-7082. These results suggested that TNF-α upregulated ICAM-I via an NF-κB–dependent pathway in HUVECs to a far greater extent than adiponectin. In addition, we examined the synergistic effects of adiponectin and TNF-α on ICAM-1 expression. Unexpectedly, the induction of ICAM-1 by TNF-α was inhibited by further addition of adiponectin (Fig. 4*C*), indicating that TNF-α and adiponectin antagonized each other via the signaling pathways of these agents. This phenomenon, which was reported previously (7,24), was also observed in the actions of adiponectin and palmitate on ICAM-1 expression. Next, we examined the effects of these NF-κB activators on FHC expression in HUVECs. Although FHC expression was slightly increased by TNF-α, neither adiponectin nor palmitate treatment produced significant increases (Fig. 4*D*). Taken together, these observations indicated that induction of FHC by adiponectin does not occur in HUVECs despite the NF-κB activation, presumably due to the minor effect of adiponectin on NF-κB activation.

Recombinant FHC reduces ROS production induced by oxidative stress. To investigate the cytoprotective effects of FHC against forms of damage mediated by oxidative stresses or inflammatory cytokines, we transfected adenovirus expressing recombinant FHC into HUVECs and C2C12 myocytes. As the murine primary cells exhibited susceptibility to adenovirus infection, we performed this experiment with C2C12 myotubes. Before the ROS assay, we confirmed recombinant FHC to be overexpressed by immunoblotting using anti-murine FHC antibodies. With overexpression of recombinant FHC, 13 and 28% reductions in relative ROS accumulations were observed in HUVECs and C2C12 myotubes, respectively (Fig. 5). In addition, FHC had a major effect on reducing ROS accumulation induced by various forms of oxidative stress (i.e., 26% [Fe^{2+}], 18% [TNF-α], and 25% [high glucose] in HUVECs; and 26% [(Fe^{2+}], 20% [TNF-α], and 49% [4-hydroxynonenal] in C2C12 myotubes), indicating that FHC exerts cytoprotective effects by reducing the ROS accumulation induced by various oxidative stresses. When these cells were treated with adiponectin, ROS-reducing effects, which were similar to those obtained with FHC overexpression, were also observed (data not shown). Taken together, these findings indicated FHC upregulation by adiponectin in skeletal muscle cells to at least partially explain the ROS-reducing effects of adiponectin.

Increased expression of NF-κB target genes with adiponectin incubation and their contributions to the ROS-reducing effects of adiponectin. We further investigated NF-κB target gene expressions (e.g., MnSOD and iNOS) by quantitative PCR. As shown in Fig. 6, these proteins (FHC, MnSOD, and iNOS) were similarly upregulated by adiponectin incubation (3.6-, 1.6-, and 5.1-fold, respectively, in skeletal muscle cells and 4.3-, 1.8-, and 3.5-fold, respectively, in C2C12 myotubes). No MnSOD protein was identified on our two-dimensional electrophoresis search for proteins upregulated more than three-

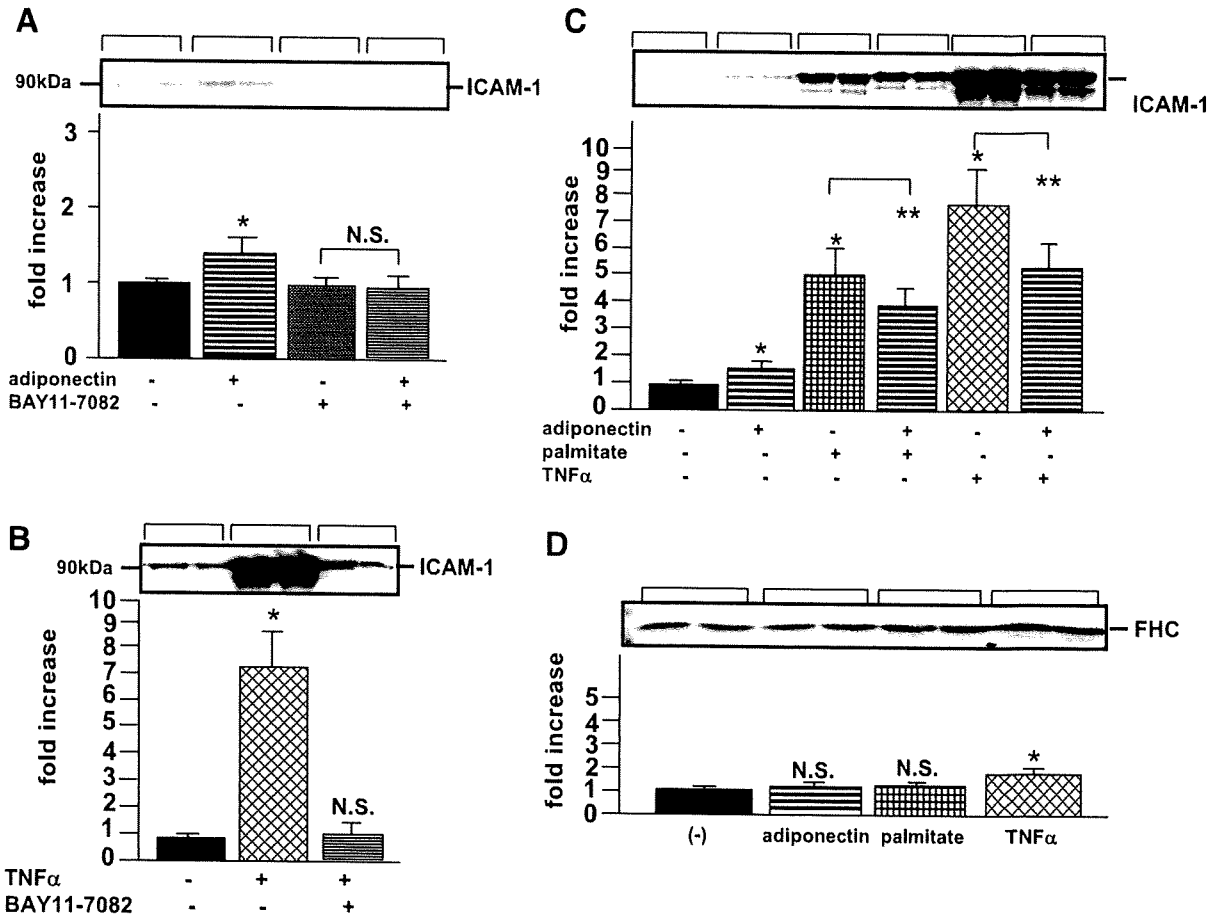

FIG. 4. Effects of recombinant human adiponectin, TNF-α, and free fatty acids on NF-κB–regulated gene expressions in HUVECs. After pretreating HUVECs with or without 100 μmol/l BAY11-7082 for 1 h, 25 μg/ml of adiponectin (*A*) or 10 ng/ml of TNF-α (*B*) were added, followed by incubation for 12 h. After HUVECs had been pretreated with or without 25 μg/ml of adiponectin for 1 h, 10 ng/ml of TNF-α or 0.4 mmol/l palmitate was added, followed by incubation for 12 h (*C* and *D*). Cell lysates from HUVECs were subjected to SDS-PAGE, followed by electrophoretic transfer to a nitrocellulose membrane. Membranes were incubated for 1 h at room temperature with antibody against ICAM-1 (*A*, *B*, and *C*) or FHC (*D*). Detection was performed using an electrochemiluminescence chemiluminescent kit according to the manufacturer's instructions. Representative data (each bracket) from four independent experiments (two samples for each experiment) are presented. Values are means ± SE. *Significant difference ($P < 0.05$) relative to ICAM-1 (*A–C*) and FHC (*D*) expressions in control cells in the absence of ligands. **Significant difference ($P < 0.05$) relative to ICAM-1 expression (*C*) in paired control cells in the absence of adiponectin but in the presence of palmitate (*lane 3*) or TNF-α (*lane 5*). N.S., not significant relative to FHC expression in control cells in the absence of adiponectin (*A*), TNF-α (*B*), and each ligand (*D*).

fold by adiponectin. Though the reason for our inability to identify iNOS in two-dimensional electrophoresis was not entirely clear, the minute amounts of iNOS proteins in skeletal muscles made this form of analysis impractical (25). To clarify the relevance of the observed increase in all three proteins to the changes in ROS, we investigated the ROS levels in C2C12 myotubes, in which the expressions of these three proteins were inhibited by induction of siRNAs. As shown in Fig. 7, under the condition in which no significant increases in the three gene products were observed with adiponectin incubation, we investigated adiponectin-induced changes in ROS accumulation. When FHC siRNAs were induced, we observed a marked decrease in the ROS-reducing effects of adiponectin incubation. On the other hand, there was no significant change in ROS accumulation with iNOS siRNA induction, while a slight but significant decrease was observed with MnSOD siRNA induction. These results suggest that increased FHC expression has a major impact on ROS accumulation; however, this increase does not explain the entire ROS-reducing effect of adiponectin.

Increased serum adiponectin upregulates FHC in skeletal muscles in vivo. To further confirm the FHC upregulation by adiponectin in in vivo experiments, we prepared mice expressing recombinant adiponectin by systemic adenovirus injection into the tail vein. Adenovirus gene transfer revealed ectopic overexpression of adiponectin in the liver to markedly upregulate serum adiponectin (control construct: 14.4 ± 0.6 μg/ml and adiponectin construct: 44.5 ± 4.9 μg/ml) (Fig. 8*B*). In particular, mainly the HMW and LMW forms of adiponectin were increased (Fig. 8*C*). As shown by immunoblotting of skeletal muscles, FHC expression in these muscles was increased 2.5-fold in adiponectin-transferred mice (Fig. 8*A*) (i.e., in vitro experiments confirmed FHC upregulation under physiological conditions).

DISCUSSION

Intensive previous studies (5,8) revealed adiponectin to improve insulin sensitivity and increase fatty acid oxidation in skeletal muscles. In fact, MMW or HMW adiponectin exerts these effects on skeletal muscles by activating

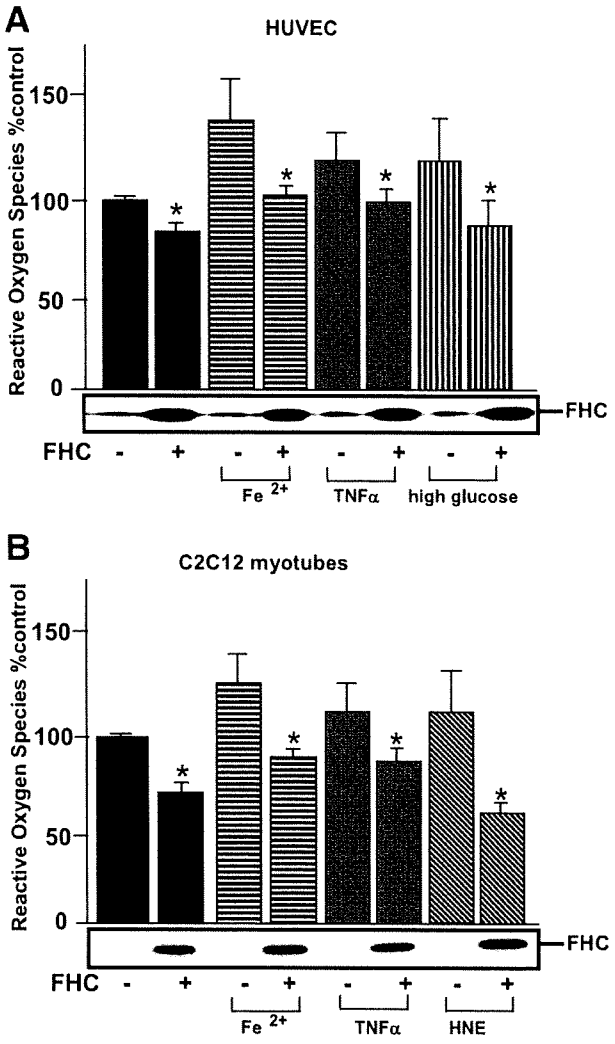

FIG. 5. Effects of recombinantly overexpressed FHC on ROS generation in C2C12 myotubes and HUVECs exposed to oxidative stresses. *A*: HUVECs, transfected with adenovirus overexpressing LacZ or recombinant FHC, were treated with 5.4 × 10^{-9} mol/l iron (II) sulfate heptahydrate and 25 mmol/l glucose. *B*: C2C12 myotubes, transfected with adenovirus overexpressing LacZ or recombinant FHC, were treated with 20 ng/ml of TNF-α and 100 μmol/l 4-hydroxynonenal (HNE). After a 24-h incubation, ROS generations were assayed by CM-H_2DCFDA oxidation-based fluorescence. Representative results from three independent experiments are presented. Values are means ± SE. *Significant difference (P < 0.05) relative to ROS production by paired control cells in the absence of FHC overexpression.

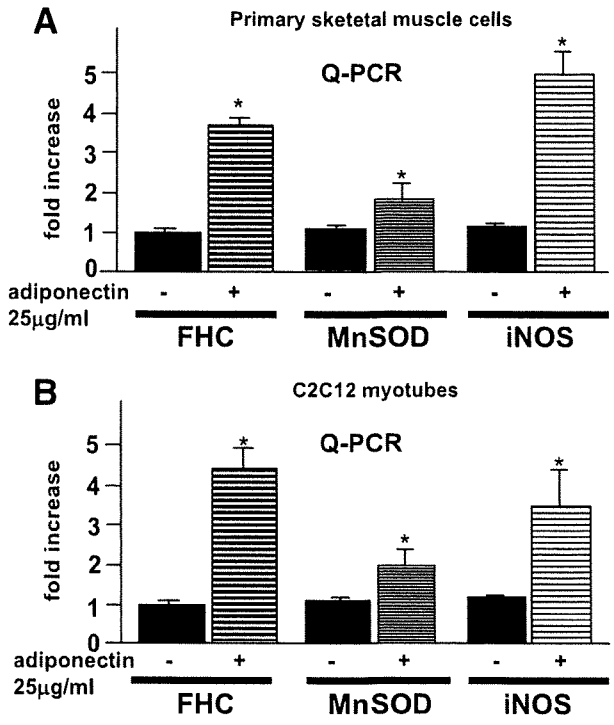

FIG. 6. FHC, MnSOD, and iNOS mRNA levels in primary skeletal muscle cells (*A*) and C2C12 myotubes (*B*) were determined by quantitative PCR using an ABI PRISM model 7000 according to the manufacturer's instructions. Each column shows the mean ± SE obtained from four samples in the presence or absence of adiponectin. *Significant difference (P < 0.05) relative to control cells in the absence of adiponectin.

AMP-activated protein kinase and peroxisome proliferator–activated receptor-α (26). On the other hand, HMW adiponectin was previously reported to activate NF-κB (10), the master coordinator of immunity, inflammation, differentiation, and cell survival (17,27–29). However, the physiological role of NF-κB activation in skeletal muscle cells has yet to be elucidated. In the present study, we treated murine primary cultured skeletal muscle cells with recombinant adiponectin and found FHC to be significantly increased. The two-dimensional gel electrophoresis–based proteomic approach used herein is generally acknowledged to be relatively insensitive in that it measures only a limited subset of tissue proteins and systematically excludes several classes. Adiponectin-induced FHC upregulation was seen only in cultured skeletal muscle cells, not endothelial cells (i.e., HUVECs). Judging from the small increase in ICAM-1 expression with adiponectin incubation, the NF-κB–activating effect of adiponectin was likely to be so small that we were unable to demonstrate FHC upregulation in HUVECs. These response differences between skeletal muscle and endothelial cells may be explained by variations in adiponectin species or different tissue localizations of molecules involved in adiponectin signaling, such as adiponectin receptors.

Our results demonstrate NF-κB activation to be involved in FHC upregulation by adiponectin. This mechanism of FHC upregulation is supported by the following data. First, in agreement with prior studies (10), we demonstrated that adiponectin does, in fact, really phosphorylate and degrade IκB-α in cultured skeletal muscle cells, thereby enhancing NF-κB activation. Second, FHC is regulated by NF-κB activation in response to enhanced oxidative stress (30). Third, FHC induction in response to adiponectin incubation is completely inhibited by BAY11-7082, an inhibitor of IκB-α phosphorylation, or NF-κB SN50, an inhibitor of NF-κB translocation into the nucleus. Though cAMP-dependent induction of FHC was previously demonstrated in human HeLa cells (31), our results show clearly that the cAMP/PKA pathway is not involved in FHC upregulation by adiponectin. Further study is needed to clarify the precise mechanisms by which FHC is regulated.

Ferritin is a major intracellular iron-storage protein that sequesters excess free iron molecules to minimize the generation of iron-catalyzed ROS (30,32). Ferritin consists of two subunits, FHC and FLC (15), and there are functional differences between these subunits. FHC has ferroxidase activity (i.e., the oxidation of Fe^{2+} to Fe^{3+}), which is involved in rapid iron uptake and release and is

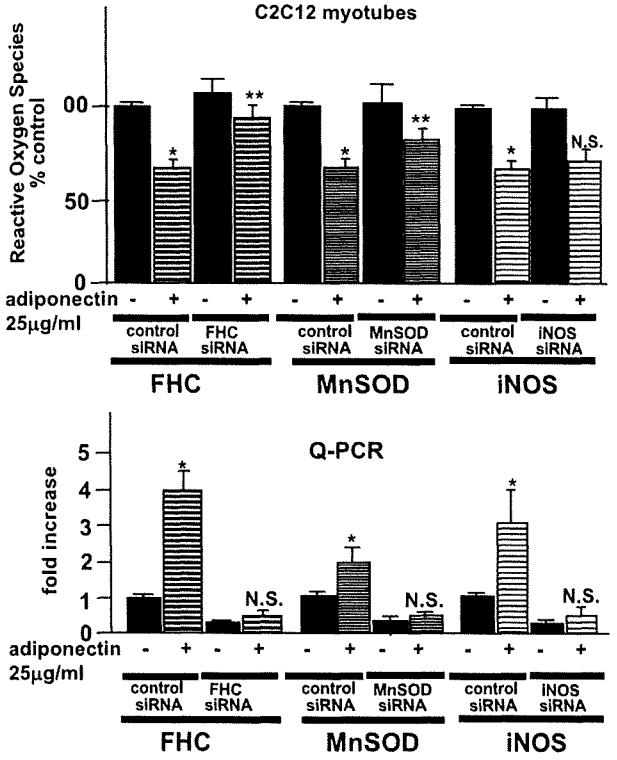

FIG. 7. C2C12 myotubes were transfected with the control nonsilencing siRNA and siRNA against FHC, MnSOD, and iNOS using the transfection reagent. The cells were used for experiments 48 h after siRNA transfection and incubated with or without recombinant adiponectin at 12 h before the experiments. ROS generations and FHC, MnSOD, and iNOS mRNA in each of the cells were determined. Each column shows the mean ± SE obtained from four samples in the presence or absence of adiponectin. *Significant difference ($P < 0.05$) relative to the paired control cells in the absence of adiponectin. **Significant difference ($P < 0.05$) relative to control siRNA-transfected cells in the presence of adiponectin. N.S., not significant relative to paired control cells in the absence of adiponectin.

required for iron sequestration (16). On the other hand, FLC has no ferroxidase activity but is likely to contribute to stabilization of assembled ferritin proteins for long-term iron storage (33). AP-1 motifs or NF-κB–responsive elements have been found in the promoter region of FHC (22,34) but not in that of FLC. Indeed, our results revealed only the expression of FHC (i.e., not that of FLC) to be increased in response to adiponectin exposure. In addition to this iron-mediated regulation, ferritin is regulated by immune/inflammatory cytokines. For example, similar changes in FHC-to-FLC ratios (i.e., increased FHC with no significant change in FLC expression) were observed in myoblasts following cytokine stimulation (35). Serum levels of ferritin were previously reported to be increased in patients with nonalcoholic steatohepatitis (36) or type 2 diabetes with obesity (37), disorders characterized by inflammation in the liver or adipose tissues. In these conditions, NF-κB plays a pivotal role in cytokine-induced FHC upregulation (17).

In recent decades, studies of NF-κB have concentrated mainly on discovering the molecules and biochemical processes essential to the signaling cascades controlling NF-κB activity, since NF-κB is known to be one of the critical transcription factors mediating inflammatory cellular responses, such as the production of cytokines and adhesion molecules (36,38). However, recent studies have focused on elucidating the novel mechanism whereby NF-κB exerts a protective effect against cytotoxicity. Notably, Pham et al. (17) discovered how NF-κB antagonizes TNF-α–induced apoptosis. They identified FHC as a critical mediator of NF-κB protective activity against TNF-α–induced cytotoxicity and concluded that FHC mediates suppression of ROS accumulation, which in turn prevents persistent activation of the Jun NH_2-terminal kinase pathway, thereby inhibiting apoptosis. Thus, in adiponectin-sensitive tissues, such as skeletal muscle cells, FHC upregulation induced by adiponectin plays a pivotal role in the antagonistic cross-talk between the NF-κB and ROS/Jun NH_2-terminal kinase pathways.

The physiological roles of NF-κB in skeletal muscles are currently unknown despite the well-recognized increase in NF-κB activity with acute exercise and muscle contraction (39). However, given the array of NF-κB target gene products in skeletal muscles, NF-κB is speculated to

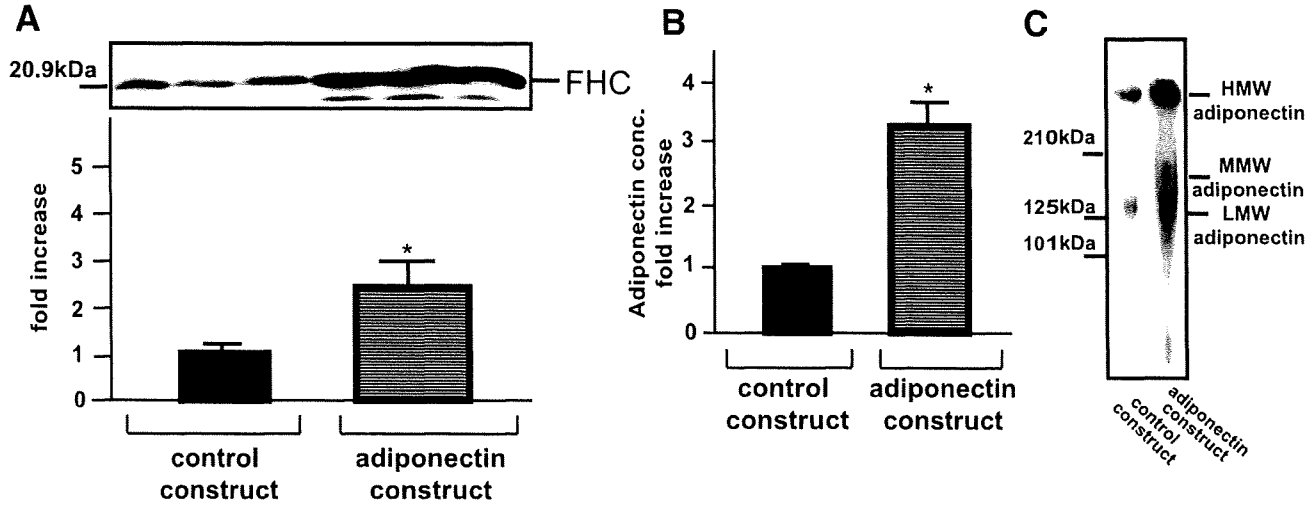

FIG. 8. Effects of adiponectin overexpression on FHC regulation in skeletal muscles. Mice were systemically injected with recombinant adenovirus expressing LacZ (L group) or adiponectin (A group) via the tail vein. Three days after virus infection, we confirmed increased serum adiponectin levels, using a mouse/rat adiponectin enzyme-linked immunosorbent assay kit (*B*) and by immunoblot analysis (*C*) and quantitatively analyzed FHC expression in total hindlimbs (*A*), as previously described (19). Representative data of three mice (*A*) or one mouse (*C*) from each group are presented. Each column shows the means ± SE obtained from seven animals in each group. *Significant difference ($P < 0.05$) relative to L group.

serve as a scavenger in states of oxidative stress because stress factors accumulate with exercise and muscle contraction (40). Along with FHC, MnSOD and iNOS were demonstrated to be increased and to be targets of the NF-κB gene product. Thus, our results suggest adiponectin to support NF-κB activation and thereby produce beneficial effects in skeletal muscle by reducing ROS via FHC upregulation.

In conclusion, we have clarified that NF-κB–targeted genes were upregulated by adiponectin in skeletal muscle cells, making this report, to our knowledge, the first ever demonstration of this property of adiponectin. Taking into consideration that ROS activity is subject to negative feedback regulation by NF-κB, adiponectin plays key roles in reducing oxidative stress and in cytoprotection against ROS in skeletal muscles. In fact, previous studies (41,42) have demonstrated a close relation between oxidative stress and insulin resistance. Thus, ROS production, following the accumulation of excessive fat, may account for the link between obesity and insulin resistance. Considering the beneficial effects of adiponectin on obesity-linked insulin resistance, FHC upregulation by adiponectin may play an important role in the mechanism by which adiponectin improves insulin resistance.

ACKNOWLEDGMENTS

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No potential conflicts of interest relevant to this article were reported.

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Metabolic Information Highway: Interorgan Metabolic Communication Via the Autonomic Nervous System

Hideki Katagiri

Introduction

Our research goals are the development of therapeutic strategies for diabetes mellitus.

The incidence of obesity is rising at an alarming rate in much of the world [1]. Obesity, especially visceral obesity, is prone to be associated with hypertension, glucose intolerance, and dyslipidemia, collectively termed the metabolic syndrome. Independently of hypercholesterolemia, especially increased levels of oxidized low density lipoproteins [2], the metabolic syndrome increases the risk for atherosclerosis and cardiovascular morbidities [1]. To overcome obesity-related diabetes and the metabolic syndrome, it seems to be necessary to treat obesity itself. Therefore, we have attempted to unravel and manipulate the regulatory systems governing body weight as well as energy metabolism.

Leptin and Leptin Resistance

What endogenous mechanisms do we have for energy homeostasis?

Leptin, one of the adipokines, is a major contributor to energy homeostasis. Leptin is secreted mainly from adipocytes into the circulating blood, in proportion to fat stores, and binds to its receptor in the hypothalamus, leading to decreased expression of orexic neuropeptide (NPY) and increased expression of anorexic neuropeptide (POMC), resulting in suppression of food intake. Therefore, when energy storage is increased, leptin secretion is increased, leading to suppression of food intake and thus weight reduction. Conversely, decreased energy storage in adipose tissue decreases leptin secretion, resulting in weight gain. Through this mechanism, the leptin system contributes to fixing body weight within a certain range [3].

H. Katagiri
Division of Advanced Therapeutics for Metabolic Diseases, Center for Translational and Advanced Animal Research, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan

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However, this system is impaired in the obese state. According to obesity development, food intake amounts are gradually increased [4]. Thus, obesity itself further enhances food intake, forming a vicious cycle for further worsening obesity. Such obesity-enhanced food intake is explained by decreased hypothalamic sensitivity to leptin. In the obese state, circulating leptin concentrations are high, but the responses of hypothalamic cells to leptin are markedly impaired. This impairment of leptin responses is called leptin resistance. Therefore, hypothalamic leptin resistance is an important mechanism maintaining obesity and a potential target for fighting obesity.

Interorgan Metabolic Communication

Then, what endogenous mechanisms other than the leptin system do we have for energy homeostasis, especially energy expenditure?

Metabolism does not go on independently in different tissues and organs, but rather in a coordinated and regulated manner throughout the body. This coordinated metabolic regulation involving several organs/tissues requires interorgan communication systems. Because the human body is a multiorgan entity, I believe that the metabolic communication network among organs is essential for metabolic homeostasis.

Humoral factors, such as insulin and adipokines, are known to play important roles in this communication. However, using tissue-specific transgenic and knockout mice, unexpected metabolic phenotypes are reported in remote tissues other than the gene-disrupted tissue, which means the presence of as yet unknown systems for metabolic communication.

Therefore, our goal is to identify ways of (1) improving leptin sensitivity, as well as (2) other endogenous mechanisms maintaining energy homeostasis, via interorgan metabolic communication.

I looked for a strategy that would give us some insight into the mechanisms underlying metabolic crosstalk among organs and tissues. I suspected that, if metabolism could be acutely altered in just one organ, it would be much easier to analyze acute effects on metabolism in other remote tissues. Intervening in this step would give us an understanding of the mechanisms. In addition, the metabolic effects might be compensatory and thereby beneficial for systemic metabolism, possibly making it a potential therapeutic target. Therefore, the interorgan communication system could become a therapeutic target for the metabolic syndrome.

Therefore, we selected an adenoviral gene transfer system to express proteins that alter metabolism in a single tissue/organ of mice that had become obese and diabetic [5]. Using this strategy, in addition to well-known humoral signals, we have recently shown the importance of afferent neuronal signals for interorgan metabolic communication.

Metabolic Information Highways

Afferent Signals for Improving Hypothalamic Leptin Resistance from Visceral Adipose Tissue

As I already described, obesity induces leptin resistance. Therefore, I wondered what happens to hypothalamic leptin sensitivity when adipose tissue is acutely diminished in obese mice. As uncoupling protein (UCP)-1 is known to enhance cellular metabolism by blocking ATP synthesis in mitochondria, we expressed UCP1 in intraabdominal fat tissues of obese mice.

Direct injection of UCP1 adenovirus into the epididymal fat of mice with high fat diet-induced obesity resulted in very limited but significant expression of UCP1 in adipocytes, with no detectable expression in other tissues. Limited expression of UCP1 in intraabdominal fat suppressed overeating in mice with diet-induced obesity, suggesting improvement of leptin resistance in the hypothalamus. To examine whether leptin sensitivity truly had improved, we performed leptin tolerance testing. Administration of leptin markedly decreased food intake as compared with the control mice. In addition, although daily leptin administration did not decrease the body weight of control mice, because of leptin resistance, the body weight of UCP-1 mice did decrease quite a lot. Thus, leptin sensitivity was clearly shown to be improved by UCP1 expression in intraabdominal adipose tissues. Furthermore, UCP1 expression in visceral adipose tissue actually altered hypothalamic neuropeptide expression: NPY expression was decreased and POMC expression was increased. These results demonstrate that enhanced metabolism in intraabdominal fat tissue does indeed improve leptin sensitivity in the hypothalamus. Then, to elucidate the underlying mechanism, we attempted to intervene in intertissue communication.

Except for markedly decreased leptin levels, no adipokines were altered. Therefore, we hypothesized that neuronal signals are involved in this effect. So, we dissected nerve bundles innervating bilateral epididymal fat tissues. Ten days after nerve dissection, LacZ or UCP1 adenovirus was injected into epididymal fat tissues. Nerve dissection blocked the suppression of overeating seen in sham-operated mice. These findings suggest that neuronal signals from intraabdominal fat tissue are involved in regulating food intake [6].

Thus, visceral adipose tissue secretes leptin into the circulating blood and also regulates its own sensitivity in the hypothalamus via a neuronal pathway (Fig. 1). Food intake is likely to be precisely regulated by these dual signals from visceral adipose tissue. Our findings may provide evidence of a third key role of adipose tissue. The first one is energy storage, and the second one is adipokine secretion. This third adipose tissue role is to transmit afferent signals about energy status to the brain [7].

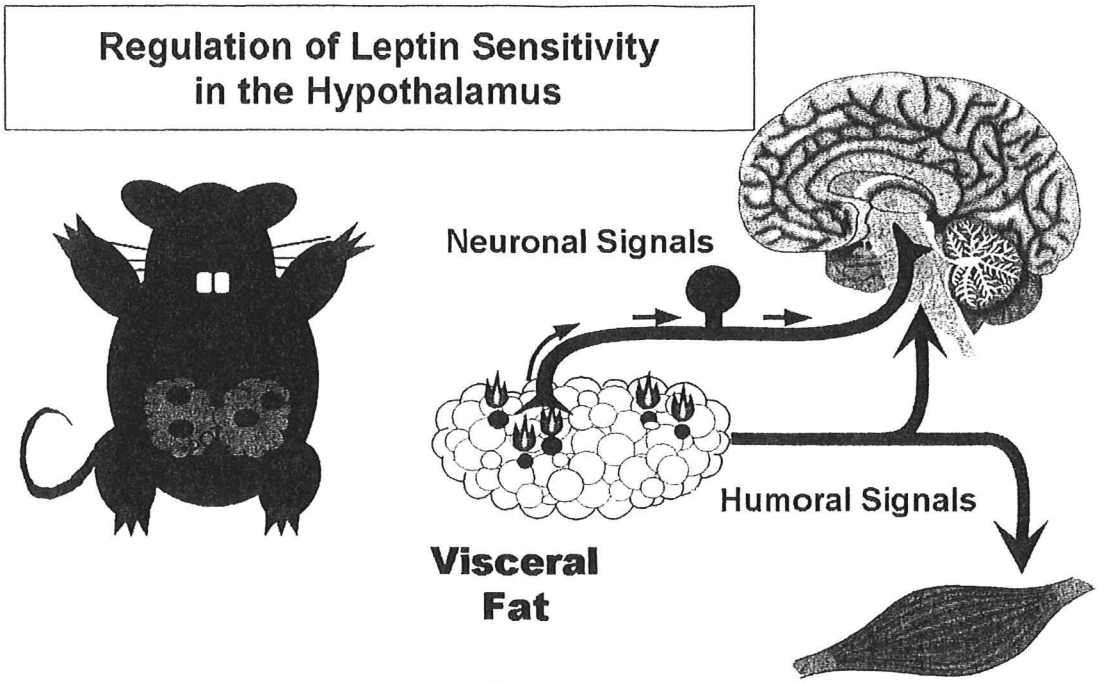

Fig. 1 Metabolic information highway from visceral adipose tissue. Neuronal signals from visceral adipose tissue regulate hypothalamic leptin sensitivity

Afferent Signals Enhancing Energy Expenditure from the Liver

We have another question: What happens when energy storage is increased in the liver?

To address this question, we attempted to express peroxisome proliferator-activated receptor-gamma (PPARγ) in the liver. PPARγ is a strong transcriptional factor that induces expression of genes involved in lipid synthesis and lipid uptake [8]. Although PPARγ expression in the liver is very low in the lean state, hepatic expression of PPARγ, especially PPARγ2, is functionally enhanced in a number of obesity models, including mice with genetically induced and high fat diet-induced obesity, as well as human obese subjects. Furthermore, liver-specific disruption of PPARγ in genetically obese mice reportedly prevents hepatic steatosis [9], suggesting PPARγ expression in the liver to play an important role in development of hepatic steatosis. Therefore, to enhance hepatic lipid accumulation, a recombinant adenovirus encoding PPARγ2 was intravenously administered. Systemic infusion of recombinant adenoviruses into mice through the tail vein resulted in PPARγ2 being expressed primarily in the liver, with no detectable expression in other tissues such as fat, muscle, or brain.

As expected, hepatic PPARγ2 expression induced severe steatosis in the liver. In contrast, intriguingly, in PPARγ2 mice, adipose tissues were remarkably diminished, by 50%, in just 1 week. Basal metabolic rates were increased, by

30%, in these mice, while food intakes were unchanged. As a result, high fat chow-induced weight gain was markedly suppressed. Surprisingly, despite marked hepatic steatosis, glucose tolerance and insulin sensitivity were markedly improved in PPARγ2 mice, as demonstrated by glucose tolerance and insulin tolerance testing.

The metabolic phenotypes associated with hepatic PPARγ2 expression are summarized here. In the liver, hepatic PPARγ2 expression induced abundant lipid accumulation. In contrast, in the periphery, fat accumulation was markedly decreased and insulin sensitivity was strikingly improved. In addition, systemic basal metabolic rates were significantly increased. As a result, systemic glucose tolerance was remarkably improved by hepatic PPARγ2 expression. These remote effects are very beneficial for fighting obesity and diabetes.

We obtained data suggesting the involvement of sympathetic nerve activation in increased lipolysis and energy expenditure. Therefore, the target of hepatic signals is not in the periphery, but rather the brain. At this time as well, we speculated about neuronal involvement. To determine whether the beneficial remote effects involve the vagal nerve, we dissected its hepatic branch.

Seven days after selective hepatic vagotomy, recombinant adenovirus was administered. In mice subjected to hepatic vagotomy, hepatic PPARγ2 expression increased liver weight and hepatic triglyceride content essentially as it had in sham-operated mice. Thus, the hepatic phenotypes are not affected by hepatic vagotomy.

In contrast, intriguingly, the decreases in brown adipocyte sizes and white adipose tissue weight as well as increases in serum free fatty acids (FFA) levels, which reflect increased lipolysis, and basal metabolic rates were completely blocked by selective hepatic vagotomy. In addition, pharmacological afferent blockade of the hepatic vagus similarly blocked these remote tissue effects.

Taken together, these observations show that hepatic PPARγ2 expression conveys metabolic information to the brain via the afferent vagus, resulting in efferent sympathetic activation, which enhances lipolysis and energy expenditure and improves obesity-related insulin resistance and diabetes. Hepatic PPARγ expression is physiologically induced when energy intake is excessive. Therefore, it may function as a feedback mechanism against excess energy intake, preventing the development of obesity by enhancing energy expenditure [10] (Fig. 2). This concept was introduced as the Metabolic Information Highway in "This Week" in *Science*, the issue in which our article was published. The autonomic nervous system is the pavement for this highway.

Let us compare this machinery with the leptin system. In the leptin system, adipose tissue recognizes excess energy accumulation and releases leptin to the brain via circulating blood. On the other hand, with this neuronal machinery, the liver senses energy storage and releases signals to the brain via the afferent vagus. While leptin mainly suppresses appetite, hepatic neuronal signals mainly enhance energy expenditure. Both inhibit weight gain. We are now working to identify which molecules activate the vagal nerve and what portions of the brain integrate peripheral metabolic signals.

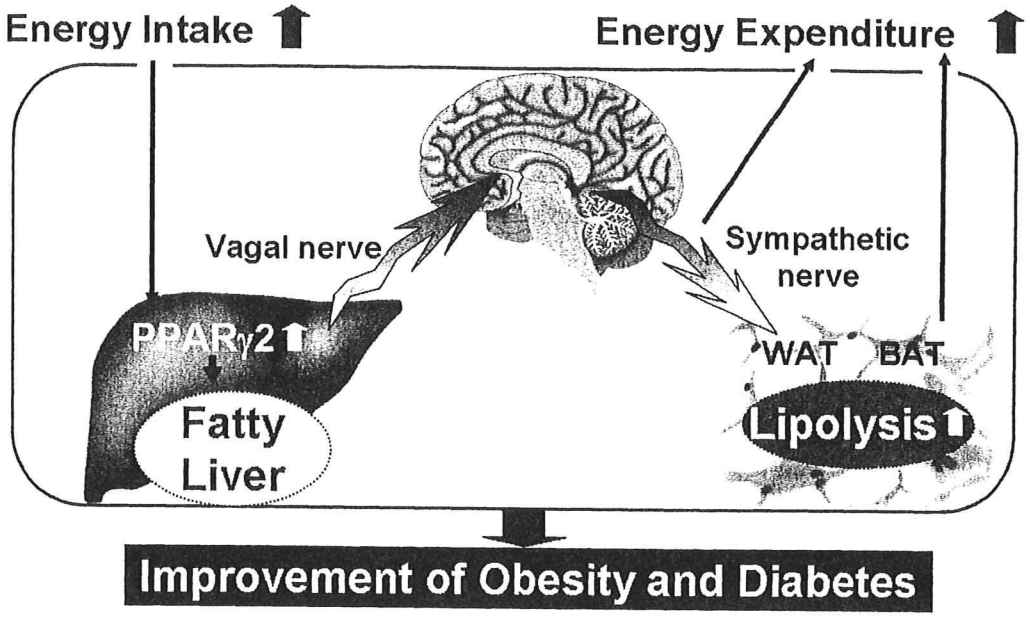

Fig. 2 Metabolic information highway from the liver. Neuronal signals from the liver regulate systemic energy metabolism to prevent the development of obesity by enhancing energy expenditure. *PPARγ*, peroxisome proliferator-activated receptor-gamma; *WAT*, white adipose tissue; *BAT*, brown adipose tissue

Conclusion

Two metabolic information highways via afferent neuronal pathways are presented: neuronal signals from adipose tissue affect hypothalamic leptin sensitization and neuronal signals from the liver regulate systemic energy expenditure. In addition to these two systems, we are now identifying another metabolic information highway from the liver to the pancreas [11]. Furthermore, the afferent signals originating in hepatic PPARγ2 expression appear to be involved in the development of obesity-related diseases (Uno K. and Katagiri H. et al., unpublished data). Thus, growing evidence has revealed the important roles of afferent neuronal signals in interorgan metabolic communication.

Collectively, the involvement of afferent neuronal signals highlights the importance of the central nervous system. The brain receives various forms of metabolic information from peripheral organs/tissues via two avenues, humoral factors and neuronal signals. These inputs from the periphery are probably integrated and processed in the brain, leading to the transmission of regulatory signals for appropriate systemic responses [1]. In addition, humoral and neuronal signals affect each other, as exemplified by the findings that adiponectin expressions are regulated by sympathetic activity [12]. Further elucidation of these regulatory systems may facilitate unraveling the mechanisms underlying metabolic homeostasis and development of the metabolic syndrome as a state of dysregulation. Moreover, targeting of the coordinated regulatory system is a potential therapeutic strategy for several types of diabetes as well as the metabolic syndrome [13].

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Article Addendum

Neural relay from the liver induces proliferation of pancreatic β cells

A path to regenerative medicine using the self-renewal capabilities

Hideki Katagiri,[1,*] Junta Imai[2] and Yoshitomo Oka[2]

Division of [1]Advanced Therapeutics for Metabolic Diseases; Center for Translational and Advanced Animal Research; and [2]Molecular Metabolism and Diabetes; Tohoku University Graduate School of Medicine; Sendai, Japan

Key words: inter-organ metabolic communication, neural relay, insulin, pancreatic β cells, regenerative medicine, diabetes, hyperinsulinemia, insulin resistance

Systemic homeostasis requires coordinated metabolic regulation among multiple tissues/organs via inter-organ communication. We have reported that neuronal signaling plays important roles in this inter-organ metabolic communication. First, we found that liver-selective extracellular signal-regulated kinase (ERK) activation induces insulin hypersecretion and pancreatic β cell proliferation. Denervation experiments revealed that these inter-organ (liver-to-pancreas) effects are mediated by a neural relay consisting of splanchnic afferents (from the liver) and vagal efferents (to the pancreas). The central nervous system also participates in this inter-organ communication. This neural relay system originating in the liver is physiologically involved in the anti-diabetes mechanism whereby, during obesity development, insulin hypersecretion and pancreatic β cell hyperplasia occur in response to insulin resistance. This indicates the pathophysiological importance of this system in diabetes prevention and hyperinsulinemia development. Furthermore, when applied to mouse models of insulin-deficient diabetes, both type 1 and type 2, hepatic activation of ERK signaling increased pancreatic β cell mass and normalized blood glucose. Thus, this inter-organ system may serve as a valuable therapeutic target for diabetes by regenerating pancreatic β cells. The concept that manipulation of an endogenous mechanism can regenerate a damaged tissue in vivo may open a new paradigm for regenerative trreatments for degenerative disorders.

*Correspondence to: Hideki Katagiri; Division of Advanced Therapeutics for Metabolic Diseases; Center for Translational and Advanced Animal Research; Tohoku University Graduate School of Medicine; 2-1 Seiryo-machi; Aoba-ku, Sendai 980-8575 Japan; Tel./Fax: +81.22.717.8228; Email: katagiri@mail.tains.tohoku.ac.jp

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In multi-organ organisms, including human beings, metabolism in different tissues and organs does not go on independently, but rather in a coordinated and regulated manner throughout the body. This coordinated metabolic regulation requires inter-organ metabolic communication and is apparently essential for maintaining systemic homeostasis, particularly glucose and energy metabolism.[1] Therefore, communication among organs/tissues is extremely important and perturbation of this control system may lead to the development of metabolic disorders. During this decade, the versatility of adipose tissue as an endocrine organ and as a contributor to disease development has been established. In this context, humoral factors, including adipokines, are known to play important roles in this communication. However, a number of recent studies have shown that tissue-specific knockout and transgenic mice exhibit unexpected metabolic phenotypes in other tissues,[2-5] suggesting the presence of as yet unknown metabolic communication systems.

Recently, several reports, including ours, have indicated that neuronal signaling, consisting of both afferent and efferent autonomic nerves, plays important roles in inter-organ metabolic communication and systemic homeostasis.[6] For instance, neuronal signals from visceral adipose tissue modulate food intake,[7] while those from the liver regulate energy expenditure.[8] In addition to these anti-obesity neuronal mechanisms, we have further identified a neuronal relay, originating in the liver, which enhances pancreatic β cell proliferation and thus functions as an endogenous anti-diabetes mechanism.

Obesity induces insulin hypersecretion and pancreatic β cell hyperplasia in response to insulin resistance. These compensatory responses of pancreatic β cells protect individuals from the development of diabetes but induce hyperinsulinemia which is involved in the pathological phenotypes of the metabolic syndrome. To elucidate the mechanisms underlying the compensatory pancreatic β cell responses, we activated proteins, which are reportedly activated in the livers of obesity models, in the livers of lean mice. Among them, hepatic signaling of extracellular signal-regulated kinase (ERK), phosphorylation of which is reportedly enhanced in the liver of a murine obesity model,[9,10] was shown to play an important role in compensatory

pancreatic β cell responses. To activate ERK in the liver, constitutively active mutant of mitogen-activated protein kinase/ERK kinase (MEK-1) was expressed in the liver using an adenoviral gene transduction system.[11] Intriguingly, liver-selective ERK activation induced insulin hypersecretion and pancreatic β cell proliferation. These pancreatic effects of hepatic ERK activation were inhibited by either splanchnic afferent blockade, pancreatic vagus dissection or midbrain transection. These results indicate that a neuronal relay system, consisting of the afferent splanchnic nerve, the central nervous system and the efferent vagus, mediates inter-organ (liver-to-pancreas) communication. In addition, blockade of this neuronal relay at each of several steps in murine obesity models inhibited pancreatic islet expansion during obesity development, showing the physiological role of this inter-organ mechanism in compensatory pancreatic β cell responses to obesity-induced insulin resistance. Furthermore, when applied to mouse models of insulin-deficient diabetes, hepatic activation of ERK signaling induced pancreatic β cell regeneration and thereby improved diabetes.

Our Study Highlights Several Novel and Important Points

First, pancreatic β cell mass was shown to be regulated by a neural relay originating in the liver. The liver is likely to sense metabolic conditions requiring insulin hypersecretion and to send signals via the neuronal information highway. This novel inter-organ mechanism may play very important roles in glucose homeostasis by regulating insulin secretion.

Second, involvement of afferent signals underscores the importance of the central nervous system in maintaining metabolic homeostasis. Afferent signals are received at the brainstem including the medulla and transferred to the secondary neurons which pass through the midbrain. Since midbrain transection blocks the pancreatic effects induced by hepatic ERK activation, the metabolic information originating in the liver is likely to be conveyed from the brainstem possibly to the diencephalon, including the hypothalamus and processed in the "metabolic center" in the diencephalon, resulting in transmission of signals inducing proliferation of pancreatic β cells via efferent nerves (Fig. 1). Thus, the brain may obtain various forms of metabolic information from peripheral organs/tissues, on a constant basis, and then transmit regulatory signals to peripheral tissues/organs throughout the body to induce appropriate systemic responses.

Third, this inter-organ machinery was shown to physiologically elicit compensatory islet responses to insulin resistance associated with obesity. These responses occur prior to hyperglycemia development, and thereby prevent diabetes during obesity development. However, this anti-diabetes mechanism induces hyperinsulinemia and in turn, ironically, contributes to development of the metabolic syndrome. Thus, this neural relay system is pathophysiologically involved in type 2 diabetes and the metabolic syndrome.

Finally, we would like to emphasize the implications and significance of therapeutic application to diabetes. Type 1 diabetes is characterized by severe pancreatic β cell loss. Decreases in pancreatic β cell mass are also reported in patients with type 2 diabetes.[12] In these patients, one potential underlying mechanism is β cell

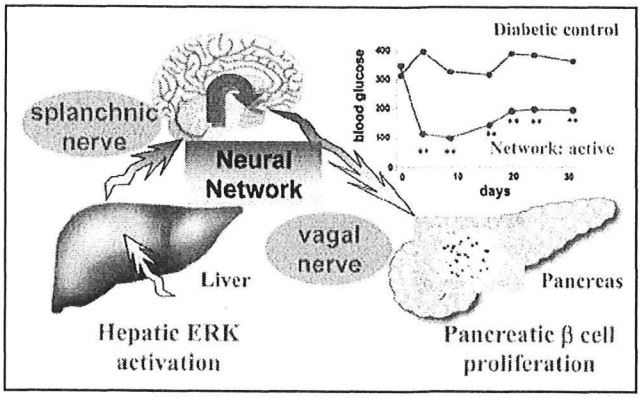

Figure 1. Schematic model of the neural relay originating in the liver (for details see text). Hepatic ERK activation associated with obesity results in pancreatic β cell proliferation, via the neuronal system consisting of afferent and efferent nerves and the central nervous system. When applied to mouse models of insulin-deficient diabetes, activation of this neural relay normalized blood glucose. This inter-organ system may serve as a valuable therapeutic target for diabetes by regenerating pancreatic β cell mass.

apoptosis induced by endoplasmic reticulum (ER) stress.[13-15] In this study, we used two mouse models of insulin-deficient diabetes, induced by severe pharmacological pancreatic β cell loss (type 1 diabetes model) and by ER stress-induced β cell apoptosis (type 2 diabetes model). In both murine models, liver-selective activation of ERK signaling resulted in an increase in β cell mass and normalization of serum glucose levels. Thus, this inter-organ system may serve as a valuable therapeutic target for diabetes, both type 1 and type 2, by regenerating pancreatic β cell mass. For regenerative medicine, many researchers are endeavoring to develop strategies whereby multi-potent cells, such as embryonic stem (ES)[16] and induced pluripotent stem (iPS)[17] cells, differentiate into an intended organ in vitro. In contrast, our study showed that stimulation of a neural machinery increased pancreatic β cells which had previously diminished. Thus, this is an example whereby manipulation of endogenous neural machinery can lead to regeneration of a damaged tissue in vivo. This concept may open a new paradigm for regenerative medicine not only for diabetes but also many other degenerative disorders.

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Cell Metabolism

Angiopoietin-like Protein 2 Promotes Chronic Adipose Tissue Inflammation and Obesity-Related Systemic Insulin Resistance

Mitsuhisa Tabata,[1,13] Tsuyoshi Kadomatsu,[1] Shigetomo Fukuhara,[7] Keishi Miyata,[1] Yasuhiro Ito,[1,2] Motoyoshi Endo,[1] Takashi Urano,[1,2] Hui Juan Zhu,[1] Hiroto Tsukano,[1] Hirokazu Tazume,[1] Koichi Kaikita,[3] Kazuya Miyashita,[8] Takao Iwawaki,[9] Michio Shimabukuro,[10] Kazuhiko Sakaguchi,[11] Takaaki Ito,[5] Naomi Nakagata,[6] Tetsuya Yamada,[12] Hideki Katagiri,[12] Masato Kasuga,[11,16] Yukio Ando,[4] Hisao Ogawa,[3] Naoki Mochizuki,[7] Hiroshi Itoh,[13] Toshio Suda,[14] and Yuichi Oike[1,15,*]

[1]Department of Molecular Genetics
[2]Department of Ophthalmology and Visual Science
[3]Department of Cardiovascular Medicine
[4]Department of Diagnostic Medicine
[5]Department of Pathology and Experimental Medicine
Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan
[6]Center for Animal Resources and Development, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan
[7]Department of Structural Analysis, National Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan
[8]Immuno-Biological Laboratories Co., Ltd., Aramachi, Takasaki 370-0831, Japan
[9]Initiative Research Unit, RIKEN Advanced Science Institute, Wako 351-0189, Japan
[10]Second Department of Internal Medicine, University of the Ryukyu, Okinawa 903-0215, Japan
[11]Division of Diabetes, Metabolism, and Endocrinology, Department of Internal Medicine, Kobe University Graduate School of Medicine, Chuo-ku, Kobe 650-0017, Japan
[12]Division of Molecular Medicine and Diabetes, Tohoku University Graduate School of Medicine, Aoba-ku, Sendai 980-8575, Japan
[13]Division of Endocrinology, Metabolism, and Nephrology, Department of Internal Medicine
[14]Department of Cell Differentiation, The Sakaguchi Laboratory
School of Medicine, Keio University, Shinjuku-ku, Tokyo 160-8582, Japan
[15]PRESTO, Japan Science Technology Agency, Kawaguchi, Saitama 332-0012, Japan
[16]Present address: Research Institute, International Medical Center of Japan, Shinjuku-ku, Tokyo 162-8655, Japan
*Correspondence: oike@gpo.kumamoto-u.ac.jp
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SUMMARY

Recent studies of obesity have provided new insights into the mechanisms underlying insulin resistance and metabolic dysregulation. Numerous efforts have been made to identify key regulators of obesity-linked adipose tissue inflammation and insulin resistance. We found that angiopoietin-like protein 2 (Angptl2) was secreted by adipose tissue and that its circulating level was closely related to adiposity, systemic insulin resistance, and inflammation in both mice and humans. Angptl2 activated an inflammatory cascade in endothelial cells via integrin signaling and induced chemotaxis of monocytes/macrophages. Constitutive Angptl2 activation in vivo induced inflammation of the vasculature characterized by abundant attachment of leukocytes to the vessel walls and increased permeability. Angptl2 deletion ameliorated adipose tissue inflammation and systemic insulin resistance in diet-induced obese mice. Conversely, Angptl2 overexpression in adipose tissue caused local inflammation and systemic insulin resistance in nonobese mice. Thus, Angptl2 is a key adipocyte-derived inflammatory mediator that links obesity to systemic insulin resistance.

INTRODUCTION

Obesity is a pandemic medical and social problem that is associated with several adverse health outcomes, including type 2 diabetes, hypertension, dyslipidemia, cardiovascular disease, and cancer (Eckel et al., 2005; Mokdad et al., 2003), all of which result in increased mortality. A major metabolic manifestation of obesity is systemic insulin resistance. Recently, the concept has emerged that chronic low-grade activation of proinflammatory pathways in adipose tissue directly promotes systemic insulin resistance (Apovian et al., 2008; Neels and Olefsky, 2006; Schenk et al., 2008). Adipocytes and macrophages could be a source of several proinflammatory cytokines that activate inflammatory pathways in resident and infiltrating cells within adipose tissue in a paracrine or autocrine fashion (Kanda et al., 2006; Weisberg et al., 2006). However, the molecular mechanisms underlying inflammation of adipose tissue in obesity have not fully clarified.

Fibrinogen promotes leukocyte adhesion and cytokine secretion at sites of inflammation through integrin-dependent inflammatory pathways (Herrick et al., 1999; Mosesson, 2005).