diabetes in animal models. Generation of insulin-producing cells from a patient's own tissues may represent a strategy for the treatment of insulin-deficient diabetes, i.e., enhancing proliferation of pre-existing β-cells by activating endogenous neural machinery. Since ERK activation might elicit undesirable effects including tumor formation, further studies are needed to develop strategies for activating this neural pathway safely and selectively, other than hepatic ERK activation.

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Case Report

Eradication of insulin resistance

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Lancet 2009; 374: 264

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In July, 2007, an 84-year-old Japanese man, with a body-mass index of 23 kg/m², presented in a cold sweat and shivering. Clinical examination was unremarkable. His blood glucose was low (3·1 mmol/L). He was not taking any medications. Platelet count (285×10⁹ per L) and HbA_{1c} (5·0%) were normal. His symptoms resolved and he was discharged. At follow-up 1 month later, his platelet count had fallen to 56×10⁹ per L and his HbA_{1c} had risen (figure). Despite treatment with several antidiabetic agents, including acarbose, pioglitazone, and metformin, HbA_{1c} increased to 9·0% 9 months after the first visit, and he was still experiencing frequent hypoglycaemic attacks. Antibodies against insulin were not detected, but antibodies against the insulin receptor were present. Our patient's serum inhibited binding of iodine-125-labelled insulin to the insulin receptor of IM-9 cells by 69·1% at a 1 to 4 dilution. Fasting plasma insulin was very high (137·9 mU/L), indicating severe insulin resistance. Type B insulin resistance syndrome was diagnosed.

In the mean time, thrombocytopenia also worsened, with the platelet count falling to 10×10⁹ per L. Antibodies against platelets (PA IgG) were detected (302 fg/platelet). Leucocytes, erythrocytes, inflammatory markers, and complement factors were all within normal limits. Bone-marrow aspirate showed no evidence of megakaryocyte hypoplasia. There were no findings suggesting collagen diseases. CT showed no evidence of pancreatic tumour, liver cirrhosis, or splenomegaly. On the basis of these findings, immune thrombocytopenic purpura (ITP) was diagnosed. *Helicobacter pylori* infection was detected by the carbon-14 urea breath test and eradication therapy (amoxicillin, lansoprazole, and clarithromycin) was given for 7 days. Following *H pylori* eradication (confirmed by breath test) platelet count increased and HbA_{1c} decreased. 6 months after *H pylori* eradication PA IgG decreased (80 fg/platelet), insulin receptor antibodies were not detectable, HbA_{1c} normalised, and hypoglycaemic episodes no longer occurred. Notably, fasting plasma insulin decreased to 10·1 mU/L, confirming striking improvement of insulin resistance. When last seen in February, 2009, our patient was not taking glucose-lowering drugs; anti-IR antibodies were still undetectable, and HbA_{1c} was normal (4·8%). Our patient had not had any new hypoglycaemic symptoms.

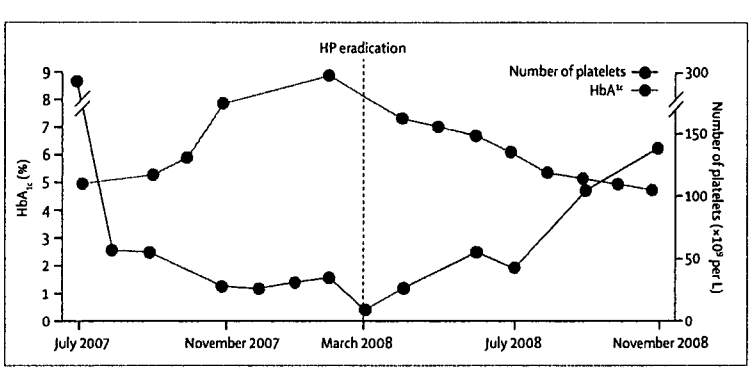

Figure: Clinical course

Type B insulin resistance syndrome is a rare cause of diabetes with severe insulin resistance and is caused by polyclonal immunoglobulin G antibodies directed against the insulin receptor. These antibodies block insulin binding to the receptor, resulting in hyperglycaemia. Paradoxically, hypoglycaemia, particularly while fasting, is occasionally associated with this disorder. Type B insulin resistance syndrome is frequently associated with other autoimmune diseases.[1] Our patient's clinical course strongly suggests that type B insulin resistance syndrome and ITP developed simultaneously and that both improved with *H pylori* eradication, which is the recommended treatment for ITP.[2] In this case, *H pylori* eradication also ameliorated type B insulin resistance syndrome. There is increasing evidence that *H pylori* infection is directly involved in modulating host immune responses.[3] Furthermore, *H pylori* eradication reportedly ameliorates some immunological disorders, including antiphospholipid antibody syndrome and rheumatoid arthritis.[4,5] Our case suggests an *H pylori* infection-related pathological mechanism underlying type B insulin resistance syndrome. There is no established effective therapy for type B insulin resistance syndrome. Indeed, it was very difficult to manage our patient's diabetes, which was also associated with occasional hypoglycaemia; treatment was no longer necessary after *H pylori* eradication. In cases of type B insulin resistance syndrome, testing for *H pylori* infection may be worthwhile, with a view to treating the infection if present.

Contributors

All authors were involved in caring for the patient. JI, TY, YO, and HK wrote the report. JI and TY contributed equally.

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Circulating oxidized LDL: a biomarker and a pathogenic factor

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Current Opinion in Lipidology 2009, 20:363–369

Purpose of review

Oxidized LDL (oxLDL) contributes to many atherogenic steps in the vascular wall, but the significance of oxLDL in circulating blood remains unclear. Recent progress in procedures for measuring both human and murine oxLDL has provided growing evidence of the importance of circulating oxLDL.

Recent findings

Circulating oxLDL is elevated in patients with advanced atherosclerosis, such as coronary heart disease and ischemic stroke, and also reflects early atherosclerotic changes and metabolic disorders including diabetes and obesity. In-vitro exposure to oxLDL increased mononuclear cell nuclear factor-κB activity, suggesting a pathogenic role of circulating oxLDL in exacerbation of oxidative stress. In addition, adenoviral administration of secreted scavenger receptor-A1, which functions as a decoy, suppresses foam cell formation in LDL receptor-deficient mice via a blockade of modified LDL incorporation into macrophages. Furthermore, when lectin-like oxLDL receptor-1 was ectopically expressed in the liver, circulating oxLDL was reduced, resulting in complete prevention of atherosclerotic progression in apolipoprotein E-deficient mice. Thus, circulating oxLDL impacts atherogenic formation.

Summary

The roles of circulating oxLDL in atherosclerotic pathogenesis are now attracting considerable attention. OxLDL removal from circulating blood is a promising therapeutic strategy against atherosclerosis.

Keywords

atherosclerosis, inflammation, oxidative stress, oxidized LDL, scavenger receptors

Curr Opin Lipidol 20:363–369

0957-9672

Introduction

The mechanisms underlying the pathogenesis of atherosclerosis are extremely complex and are affected by interactions among several biological pathways, including those of inflammation [1], metabolic disorders [2] and oxidative stress [3]. Oxidative modification of LDL is regarded as a key step in the formation of atherosclerosis [4]. Oxidized LDL (oxLDL) has been proposed to be involved in many atherogenic steps in the vascular wall such as endothelial dysfunction [5], migration of macrophages and smooth muscle cells [6] and release of inflammatory cytokines [7]. Importantly, oxLDL is incorporated into macrophages, leading to macrophage transformation into foam cells and atherosclerotic plaque formation [8]. Furthermore, oxLDL itself reportedly induces oxidative stress in endothelial cells, smooth muscle cells and macrophages, resulting in a vicious cycle of atherosclerotic progression [9]. Oxidation of LDL particles is thought to occur primarily in vascular walls rather than in plasma, which is strongly antioxidant enriched [10], and fully oxLDL is reportedly cleared from circulating blood mainly by hepatic Kuppfer cells [11]. However, recent progress in enzyme assay procedures has provided direct evidence of the presence of oxLDL in circulating blood [12–14]. Although the amounts of circulating oxLDL represent a very small fraction of total LDL [15], growing evidence indicates a relationship between circulating oxLDL and various pathogenic processes of cardiovascular disease [16]. Thus, the significance of circulating oxLDL has been established as a biomarker of atherosclerosis. In addition, several recent studies [17,18,19••,20••] revealed the pathogenic roles of circulating oxLDL in atherosclerosis. This review summarizes the relevant clinical studies on circulating oxLDL as a biomarker, as well as several animal studies, which raise the possibility of circulating oxLDL being a pathogenic factor in atherosclerosis.

Measurement of circulating oxidized LDL

OxLDL is a mixture of lipoproteins with various degrees of heterogeneous modifications such as oxidation of phospholipids, modification of apolipoprotein B (apoB) with malondialdehyde and aggregation of apoB.

Table 1 Procedures of sandwich ELISA for direct oxidized LDL assays

	Human oxLDL		
Oxidation-specific antibodies	DLH3	E06	4E6
Capture antibody	DLH3	MB47	4E6
Recognition	Oxidized phosphatidylcholines	Human apoB-100	Modified apoB-100
Detecting antibody	Sheep polyclonal	E06	Mouse monoclonal
Recognition	Human apoB-100	Oxidized phospholipids	Human apoB-100
Reference	[13]	[12]	[14]

	Murine oxLDL	
Oxidation specificity	LOX-1	DLH3
Capture molecule	Recombinant LOX-1	DLH3
Recognition	LOX-1 ligands	Oxidized phosphatidylcholines
Detecting antibody	Chicken monoclonal	Rabbit polyclonal
Recognition	Mouse and human apoB	Mouse apoB-48
Reference	[22]	[20••]

apo, apolipoprotein; LOX-1, lectin-like oxLDL receptor-1; oxLDL, oxidized LDL.

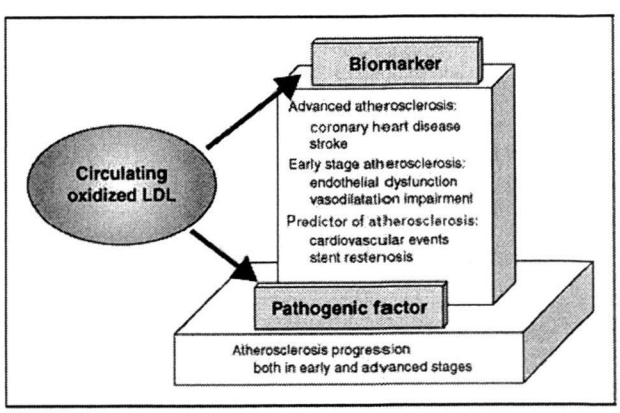
Currently, three ELISAs, using murine mAbs detecting different epitopes, details of which were described in another review [21], are widely used [12,13,22] for the measurement of circulating oxLDL (Table 1). However, these measuring methods did not allow us to analyze murine oxLDL, as antibodies against human apoB have no reactivity to murine apoB. Recently, two innovative novel immunochemical methods have been developed for measuring murine circulating oxLDL levels (Table 1). The sandwich ELISA using a chicken mAb for apoB and recombinant lectin-like oxLDL receptor-1 (LOX-1) protein, an oxLDL receptor [23], now allows measurement of circulating murine oxLDL. In another study [20••], a rabbit polyclonal antibody was raised for mouse apoB-48, a major component of murine LDL, instead of the antibody for human apoB-100, and the ELISA can be used in combination with an antibody against oxidized phospholipids, DLH3. These novel methods enabled us to measure circulating oxLDL in experimental animals, promoting active investigation of the pathogenic roles of circulating oxLDL.

Biomarker for atherosclerosis

According to oxLDL measurements in humans, evidence indicating the involvement of circulating oxLDL in cardiovascular diseases is growing [16]. The net values of these ELISA results are not comparable among studies because different antibodies detecting a variety of oxidative sites of LDL were used. However, similar associations of circulating oxLDL levels with certain pathological conditions are consistent among these clinical studies. Circulating oxLDL values were significantly elevated in patients with coronary heart disease [24], especially acute coronary syndrome [25,26]. Plasma levels of circulating oxLDL were also associated with carotid atherosclerosis [27] and ischemic stroke [28]. In addition, circulating oxLDL was increased in patients with renal failure receiving hemodialysis [14] and was shown to be a prognostic marker of cardiovascular events in cardiac transplant patients [29]. These results indicate the significance of circulating oxLDL as a biomarker of advanced atherosclerosis (Fig. 1).

Furthermore, the levels of circulating oxLDL are likely to reflect early changes promoting atherosclerosis, such as endothelial dysfunction [30] and impairment of vasodilatation responses [31,32], in individuals without apparent atherosclerosis (Fig. 1). In addition, recently, several studies have raised the possibility that circulating oxLDL levels can be used for the prediction of future cardiovascular events. Increased levels of circulating oxLDL were shown to be a strong predictor of cardiovascular events [33–35] as well as of stent restenosis in patients who had

Figure 1 The pathogenic roles of circulating oxidized LDL in atherosclerotic development may underlie the established roles of circulating oxidized LDL as a biomarker of early and advanced stages of atherosclerosis



received coronary intervention [36], although prospective studies involving a large number of patients will be needed to show circulating oxLDL to be an independent biomarker for predicting cardiovascular events.

Biomarker for metabolic disorders

Accumulating clinical observations have revealed intriguing aspects of circulating oxLDL, serving as a biomarker for metabolic disorders in addition to atherosclerosis. Several studies have demonstrated the significant elevation of circulating oxLDL values in the patients with diabetes mellitus [37] and insulin resistance [38]. Circulating oxLDL values also correlate positively with degrees of obesity [24,39–41]. Body weight reduction after gastric banding surgery decreased circulating oxLDL levels, which had been elevated in association with obesity [42]. The body weight reduction induced by dietary restriction also decreased plasma levels of circulating oxLDL in postmenopausal women [43]. Importantly, in several studies [41,43], the circulating oxLDL level showed a much stronger association with the degree of obesity than did the LDL level.

Interestingly, circulating oxLDL was reported to be positively associated with the incidence of metabolic syndrome [44]. A major factor underlying the strong relationship between oxLDL levels and metabolic disorders is considered to be the enhancement of oxidative stress in visceral adiposity. Although the mechanism underlying obesity-induced oxidative stress has not been fully elucidated, several studies [45,46] have suggested that a low degree of inflammation in obesity involves macrophage infiltration into visceral adipose tissue, leading to the increased secretion of inflammatory cytokines such as TNF-α, monocyte chemoattractant protein-1 and IL-6. Chronic inflammation exacerbates systemic oxidative stress. In addition, excess energy storage in adipose tissue may suppress expression of superoxide dismutase, which prevents oxidative stress [47]. These mechanisms together may promote oxidation of LDL particles.

Leptin, a major adipokine, appears to be involved in the greater oxidative stress in obese patients. Previous studies [48,49] demonstrated a direct effect of leptin on the generation of reactive oxygen species in endothelial cells. In fact, Porreca *et al.* [43] reported changes in circulating oxLDL values induced by body weight reductions to be strongly associated with changes in plasma leptin levels. In addition to these mechanisms, increasing oxidative stress, small dense LDL particles, which are more prone to oxidation than intermediate LDL, are reportedly increased in obese patients [50]. Taken together, these lines of evidence indicate that a high concentration of circulating oxLDL is apparently associated with metabolic disorders via various mechanisms attributable to visceral adiposity-induced oxidative stress.

Sites of LDL oxidization

Where is LDL oxidized? As circulating oxLDL temporarily increases during the acute phase of myocardial infarction or stroke, and then gradually declines to normal levels during pathological improvement [36,51], oxLDL was believed to be released from ruptured plaques into the circulation at the time of infarction occurrence. However, if the sites of LDL oxidation were limited to atherosclerotic plaques, circulating oxLDL would not be elevated in individuals without apparent atherosclerosis. Interestingly, circulating oxLDL is temporarily elevated prior to atherosclerotic progression in apoE-deficient mice [20••]. The atherosclerotic lesion area was remarkably increased from 28 to 40 weeks of age. In contrast, circulating oxLDL transiently increased at 20 weeks of age and then gradually decreased through 40 weeks of age. These results suggest that circulating oxLDL, which increases prior to atherosclerotic development, plays a pathogenic role in the early stage of atherosclerosis. Although the sites at which LDL is oxidized and those from which oxLDL is released into circulating blood are essentially unknown, arterial medial tissue under oxidative stress has been hypothesized to be a major site of LDL oxidation before and in the early stage of atherosclerosis [20••].

Indirect evidence of circulating oxidized LDL as the pathogenic factor in atherosclerosis

OxLDL is well known to be a major pathogenic factor in atherosclerotic formation, when oxLDL is localized at the vascular wall [52]. On the contrary, whether oxLDL in plasma has biological effects remains unclear. Several research groups have endeavored to explore the pathogenic roles of circulating oxLDL in the formation of atherosclerosis.

In the Watanabe heritable hyperlipidemic rabbits, plasma levels of oxLDL, which were measured as ligands for LOX-1, were higher than in control rabbits as early as 2 months of age, but antioxidant supplementation reduced plasma levels of oxLDL without altering plasma total cholesterol (TC), accompanied by suppression of atherosclerosis development. This result indirectly suggested a pathogenic role of LDL oxidation in the progression of atherosclerosis [53].

Several human studies also suggest a possible pathogenic role of circulating oxLDL. An intriguing association between circulating oxLDL and inflammation was reported in patients with angina pectoris [54]. The authors measured plasma oxLDL levels and circulating

NF-κB in peripheral blood mononuclear cells. The angina pectoris patients had higher levels of both circulating oxLDL and NF-κB activity than controls. Interestingly, in-vitro addition of either high-dose oxLDL or serum from the patients with unstable angina increased the NF-κB activity of mononuclear cells via the LOX-1 pathway. These findings suggested that NF-κB activation was induced, at least partially, by circulating oxLDL, leading to increased oxidative stress in the angina pectoris patients.

In addition, antibodies against oxLDL reported to play important roles in atherogenic regulation [55]. Antibodies to oxLDL have been found in human and rabbit plasma [56], as well as in atherosclerotic lesions of humans [57]. Most studies have shown elevated antibody titers to oxLDL, especially IgG, to be related to the degree of atherosclerotic progression. Paradoxically, though interestingly, in another study [15], circulating oxLDL levels correlated negatively to the IgG titers against oxLDL. An inverse relationship between IgM titers and atherosclerotic disease has also been reported [58,59]. In addition, inducing production of IgM antibody to oxLDL in LDL receptor-deficient mice decreased the extent of atherosclerosis [60]. Furthermore, intriguingly, treatment with a recombinant human IgG1 antibody against a malondialdehyde-modified apoB-100 peptide sequence, a specific oxLDL epitope, has been shown to reduce the level of circulating oxLDL [61] and to induce regression of preexisting lesions in LDL receptor-deficient mice overexpressing human apoB-100 [62]. These results together suggest that oxLDL antibodies play a role in maintaining low levels of circulating oxLDL. Production of immune complexes against oxLDL might prevent the development of atherosclerosis, at least partly, due to inhibition of oxLDL incorporation into macrophages [11].

Direct evidence of oxidized LDL as the pathogenic factor in atherosclerosis

To evaluate the importance of plasma-modified LDL, including oxLDL, in atherosclerotic progression, several experimental animals in which scavenger receptors were manipulated have been generated. Class A scavenger receptors (SR-A), the first cloned and now well investigated scavenger receptor family [63], play a role in the incorporation of modified LDL into macrophages, leading to foam cell formation. Whitman *et al.* [64] established the mouse model of macrophage-specific SR-A1 overexpression, and using the bone marrow transplantation technique, SR-A1 overexpression in macrophages was induced in hypercholesterolemic LDL receptor-deficient mice, resulting in inhibition of aortic atherosclerosis. However, as apoB-containing lipoproteins, including total LDL, were decreased in this study, whether intermediate LDL or modified LDL is important for the development of atherosclerosis remains unclear.

Next, to clarify the role of circulating modified LDL in the pathogenesis of atherosclerosis, Laukkanen *et al.* [17] constructed a secreted type of SR-A1 as a fusion protein consisting of a bovine growth hormone signal sequence and the extracellular domain of human SR-A1. The secreted SR-A1 functioned as a 'decoy' blocking the incorporation of modified LDL into macrophages. Adenoviral administration of secreted SR-A1 delayed the clearance of modified LDL, resulting in suppression of foam cell formation in macrophages as well as prevention of atherosclerotic lesion in LDL receptor-deficient mice [18]. Thus, blockade of the incorporation of modified LDL into macrophages may exert a beneficial effect in prevention of atherosclerosis.

Recently, we directly demonstrated the atherogenic impact of oxLDL removal from circulating blood [19••]. LOX-1 is one of the scavenger receptors and incorporates oxLDL selectively among modified types of LDL. To examine the effects of oxLDL removal from circulating blood on atherosclerotic progression, we expressed LOX-1 ectopically in the livers of apolipoprotein E (apoE)-deficient mice, using an adenoviral gene transfer system. LOX-1 expressed in the liver successfully functioned as a receptor of circulating oxLDL, thereby reducing values of circulating oxLDL, with no significant changes in plasma TC, triglyceride or LDL cholesterol (LDL-C) levels. This transient reduction in circulating oxLDL completely prevented atherosclerotic progression in apoE-deficient mice. In addition, hepatic LOX-1 expression markedly suppressed oxidative stress and inflammation in the whole body, especially in the aorta. Furthermore, smooth muscle cell deposition in the surface areas of atherosclerotic plaques was increased, possibly leading to plaque stability [19••].

These studies provide direct evidence that circulating oxLDL plays important roles in atherogenesis via mechanisms involving both direct (inhibition of foam cell formation) and indirect (antioxidative stress) effects. Thus, oxLDL removal is a promising therapeutic strategy against atherosclerosis (Fig. 1).

Reduction in circulating oxidized LDL

Then, which procedures can reduce circulating oxLDL? In general, plasma levels of circulating oxLDL correlate significantly with total levels of LDL-C [65]. Statin therapy is an established approach to decreasing LDL-C. As expected, administration of statins also reduced circulating oxLDL values, which depended on the degree

of LDL-C reduction [66–68]. However, according to the Multicenter InSync Randomized Clinical Evaluation (MIRACLE) trial [69], administration of atrovastatin to patients with coronary heart disease elevated the plasma ratio of oxLDL:apoB. Thus, oxidative phospholipids might be condensed in LDL particles after statin therapy.

Several trials [70–75] of antioxidant therapies designed to inhibit the oxidation step of LDL have been reported, but effectiveness against atherosclerosis is controversial. In murine models, administration of antioxidants effectively reduces atherosclerosis [72]. However, the majority of clinical trials yielded negative results [75]. This may at least partly be due to insufficient antioxidant effects of natural and synthetic compounds when administered to humans. For instance, in a randomized placebo-controlled study [76] in healthy adults, daily administration of high-dose vitamin E did not affect the breakdown of lipid peroxidation products. Moreover, high doses of these antioxidants reportedly have adverse effects [75], including the pro-oxidant effects of vitamin E at high doses [77]. Therefore, clinical applications of antioxidants seem to be limited at present. The development of novel strategies for lowering oxLDL itself is highly anticipated.

Conclusion

Over the past decade, investigations of circulating oxLDL have progressed dramatically. The important role of circulating oxLDL as a biomarker of cardiovascular disease has been largely established. However, further extensive examinations will be required to explore predictive values for future atherosclerotic events. In addition, the pathogenic roles of circulating oxLDL in the formation of atherosclerosis are now being elucidated. The pathogenic involvement of circulating oxLDL may account for its significance as a biomarker of not only advanced atherosclerosis but also the early-stage atherosclerosis (Fig. 1). As oxLDL may induce inflammation as well as oxidative stress, the roles of oxLDL, not only in the vascular wall but also in circulating blood, are now attracting considerable attention from investigators working on the pathogenesis of atherosclerosis. Moreover, circulating oxLDL removal is a promising strategy for the treatment of atherosclerosis.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research to H. Katagiri (B2, 15390282) and Y. Oka (A2, 19209034) from the Ministry of Education, Science, Sports and Culture of Japan and a Grant-in-Aid for Scientific Research (H19-genome-005) to Y. Oka from the Ministry of Health, Labor and Welfare of Japan. This work was also supported by the Global Centers of Excellence Program for 'Network Medicine', with funding to Y. Oka from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 428).

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ORIGINAL ARTICLE

Adiponectin Upregulates Ferritin Heavy Chain in Skeletal Muscle Cells

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OBJECTIVE—Adiponectin is an adipocyte-derived protein that acts to reduce insulin resistance in the liver and muscle and also inhibits atherosclerosis. Although adiponectin reportedly enhances AMP-activated protein kinase and inhibits tumor necrosis factor-α action downstream from the adiponectin signal, the precise physiological mechanisms by which adiponectin acts on skeletal muscles remain unknown.

RESEARCH DESIGN AND METHODS—We treated murine primary skeletal muscle cells with recombinant full-length human adiponectin for 12 h and searched, using two-dimensional electrophoresis, for proteins upregulated more than threefold by adiponectin compared with untreated cells.

RESULTS—We found one protein that was increased 6.3-fold with adiponectin incubation. MALDI-TOF (matrix-assisted laser desorption/ionization–top of flight) mass spectrometric analysis identified this protein as ferritin heavy chain (FHC). When murine primary skeletal muscle cells were treated with adiponectin, IκB-α phosphorylation was observed, suggesting that adiponectin stimulates nuclear factor (NF)-κB activity. In addition, FHC upregulation by adiponectin was inhibited by NF-κB inhibitors. These results suggest NF-κB activation to be involved in FHC upregulation by adiponectin. Other NF-κB target genes, manganese superoxide dismutase (*MnSOD*) and inducible nitric oxide synthase (*iNOS*), were also increased by adiponectin treatment. We performed a reactive oxygen species (ROS) assay using CM-H_2DCFDA fluorescence and found that ROS-reducing effects of adiponectin were abrogated by FHC or MnSOD small-interfering RNA induction.

CONCLUSIONS—We have demonstrated that adiponectin upregulates FHC in murine skeletal muscle tissues, suggesting that FHC elevation might partially explain how adiponectin protects against oxidative stress in skeletal muscles. ***Diabetes* 58:61–70, 2009**

Adipocytes have been recognized to secrete a variety of proteins, such as tumor necrosis factor (TNF)-α, adipsin, plasminogen activator inhibitor-1, leptin, resistin, and adiponectin. These proteins are termed adipokines and are likely to physiologically exert a variety of hormonal actions (1). Among these proteins, adiponectin is exclusively expressed in adipose tissue and consists of an NH_2-terminal collagenous domain and a COOH-terminal globular domain (2). Adiponectin belongs to the soluble collagen superfamily and has structural homology with collagens VIII and X, complement factor C1q (3), and the TNF family (2,4). Circulating adiponectin is extremely abundant (~15 μg/ml), and adiponectin forms various oligomeric complexes, including low (LMW), medium (MMW), and high (HMW) molecular weight species. Adiponectin exerts antidiabetes effects on muscles and the liver through AMP-activated protein kinase activation (5) and antiatherosclerotic effects by inhibiting monocyte adhesion to endothelial cells and lipid accumulation into macrophages (6,7). Thus, adiponectin increases glucose uptake and fatty acid oxidation in muscles via the type 1 adiponectin receptor (8), and decreases hepatic gluconeogenesis via the type 2 adiponectin receptor (8,9). On the other hand, nuclear factor (NF)-κB but not AMP-activated protein kinase activity was demonstrated to be enhanced by MMW or HMW adiponectin in muscles (10). According to recent studies (9–14), HMW adiponectin appears to be more important for the antidiabetes and antiatherosclerotic effects than the other two oligomeric complexes. Though the physiological role of HMW adiponectin in improving insulin resistance or reducing oxidative stress is clearly significant, the precise mechanisms by which adiponectin acts on skeletal muscles remain unknown.

Therefore, in the present study, we investigated adiponectin function in primary cultured skeletal muscle cells by comparing protein expressions in untreated cells using two-dimentional electrophoresis. A marked increase in FHC protein was observed with adiponectin incubation. FHC is one of two subunits of ferritin, the other being ferritin light chain (FLC) (15), and has ferroxidase activity, which is required for iron sequestration (16). FHC was reported to suppress reactive oxygen species (ROS) production (17), which may explain the ROS-reducing effects of adiponectin. FHC upregulation followed by an enhanced ROS-reducing effect is suggested to be a novel mechanism by which adiponectin acts directly against oxidative stress.

RESEARCH DESIGN AND METHODS

Cell culture and chemicals. Murine primary cultured skeletal muscle cells were purchased from Cell Garage (Tokyo, Japan) in cultured flasks, and maintained in DMEM containing 10% fetal bovine serum (FBS). We switched the medium to DMEM containing 2% horse serum, which promotes differentiation of myocytes into myotubes, and continued the incubation for 5 days before the experiments. Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex (Baltimore, MD) as cryopreserved cells. After thawing, the cells were plated in collagen-coated culture flasks and cultured to confluence in EBM-2 medium (Lonza, Walkersville, MD) containing the indicated ligands, 2% FBS, and antibiotics (BulletKit EGM-2, cat. no. CC-3162). C2C12 myoblasts were maintained in DMEM containing 10% FBS at 37°C in 5%

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Received 25 May 2007 and accepted 8 October 2008.
Published ahead of print at http://diabetes.diabetesjournals.org on 17 October 2008. DOI: 10.2337/db07-0690.

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, 66945), 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.

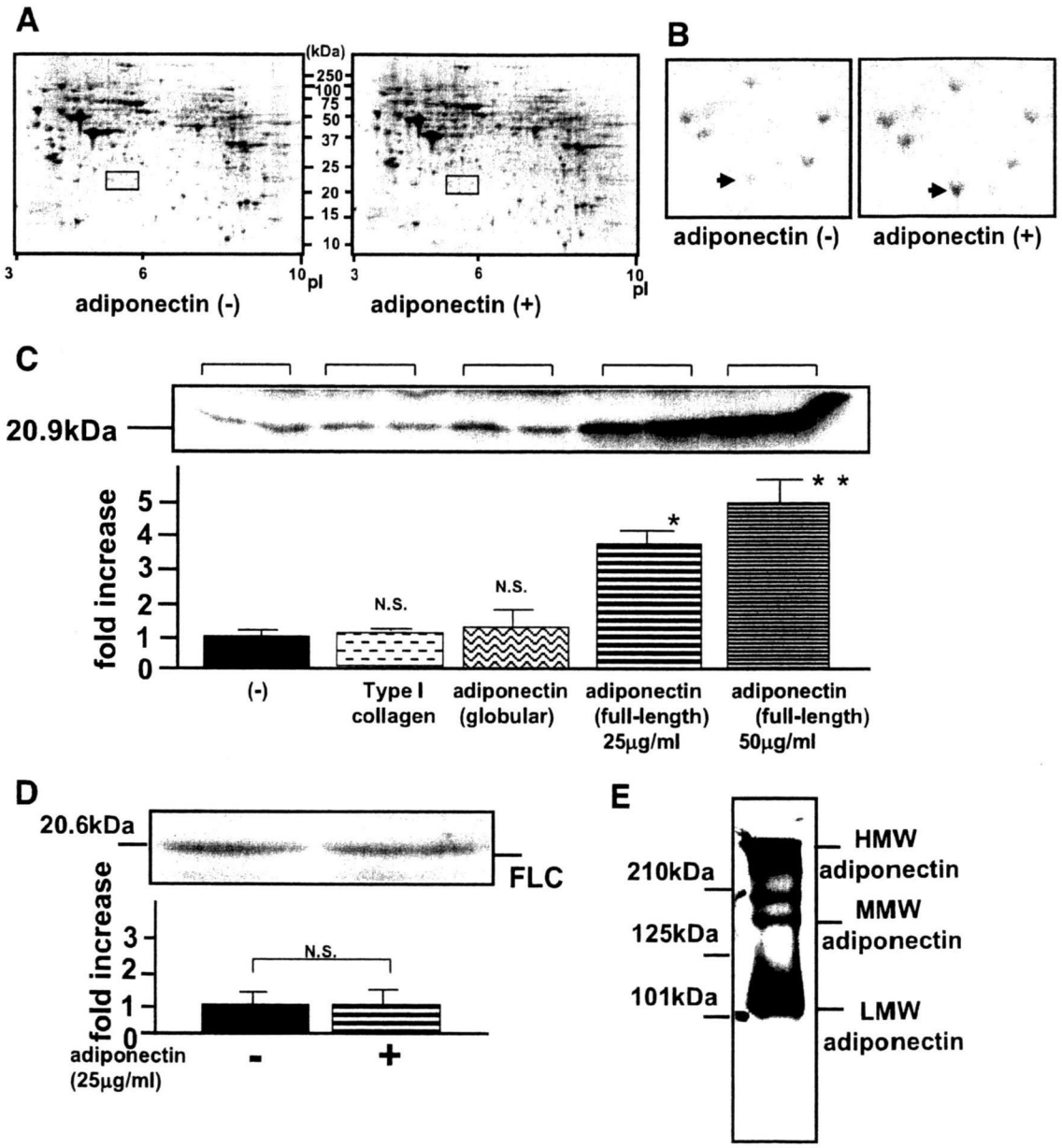

FIG. 1. Effects of incubation with HMW adiponectin on primary cultured skeletal muscles. Primary cultured murine skeletal muscles were incubated for 12 h in the presence (+) or absence (−) of adiponectin (25 μg/ml). *A*: Whole images of Coomassie brilliant blue–stained gels for two-dimensional electrophoresis 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 threefold 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

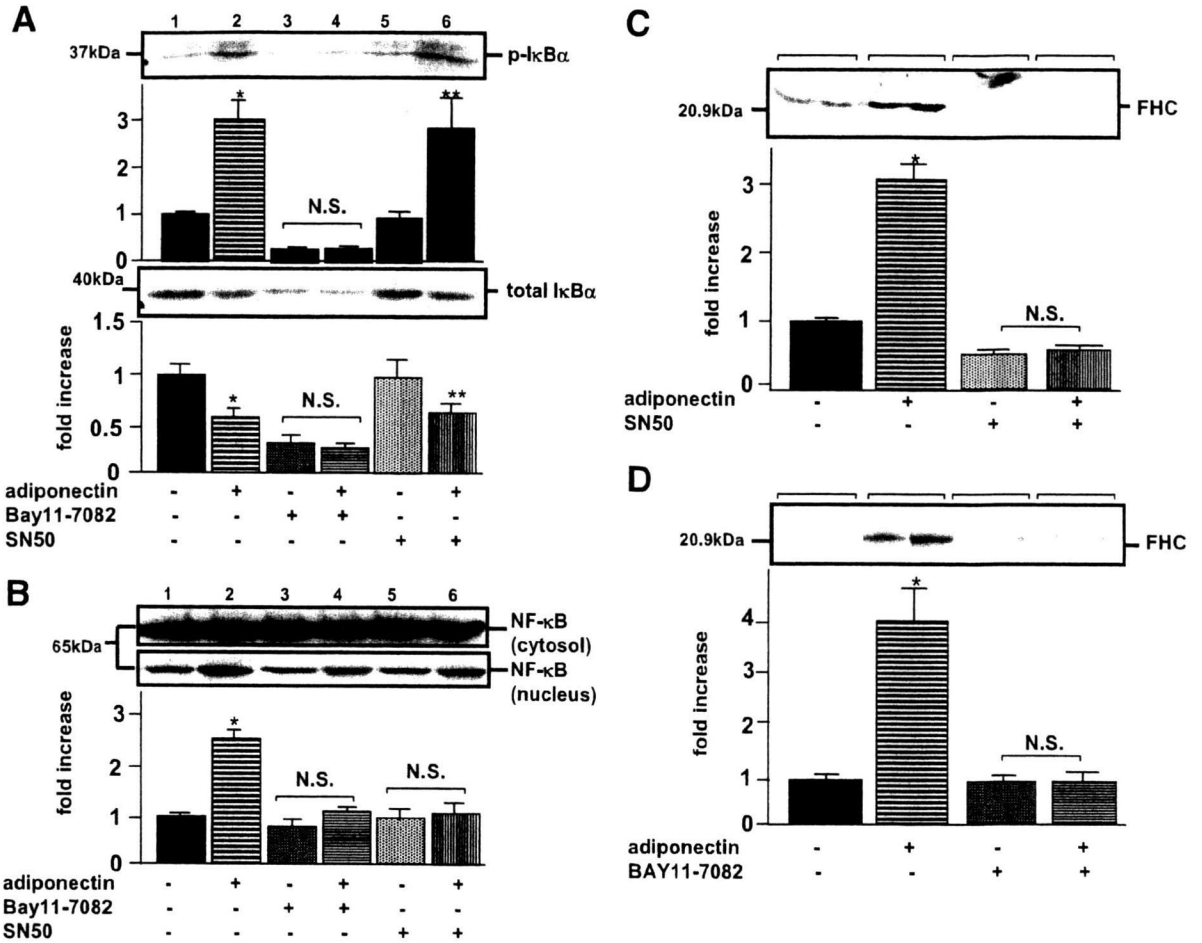

FIG. 2. Effects of IκB/NF-κB inhibitor on FHC in murine primary cultured skeletal muscle cells. Primary cultured skeletal muscle cells were pretreated with 100 μmol/l BAY11-7082 or 50 μg/ml NF-κB SN50 at 1 h before the cells were incubated with 40 μg/ml of adiponectin for 12 h. Cells were lysed in ice-cold 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-

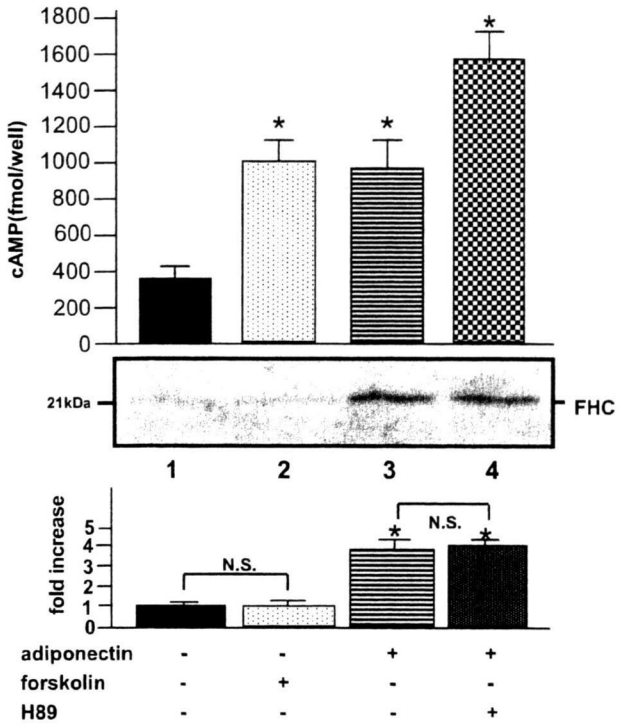

FIG. 3. Effects of recombinant human adiponectin on cAMP content of murine primary skeletal muscle cells. Primary skeletal muscle cells, cultured in 96-well plates, were exposed to 50 μg/ml of adiponectin and 5 μmol/l forskolin for 12 h. Adiponectin-treated cells were also pretreated with 1 μmol/l H89, 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-

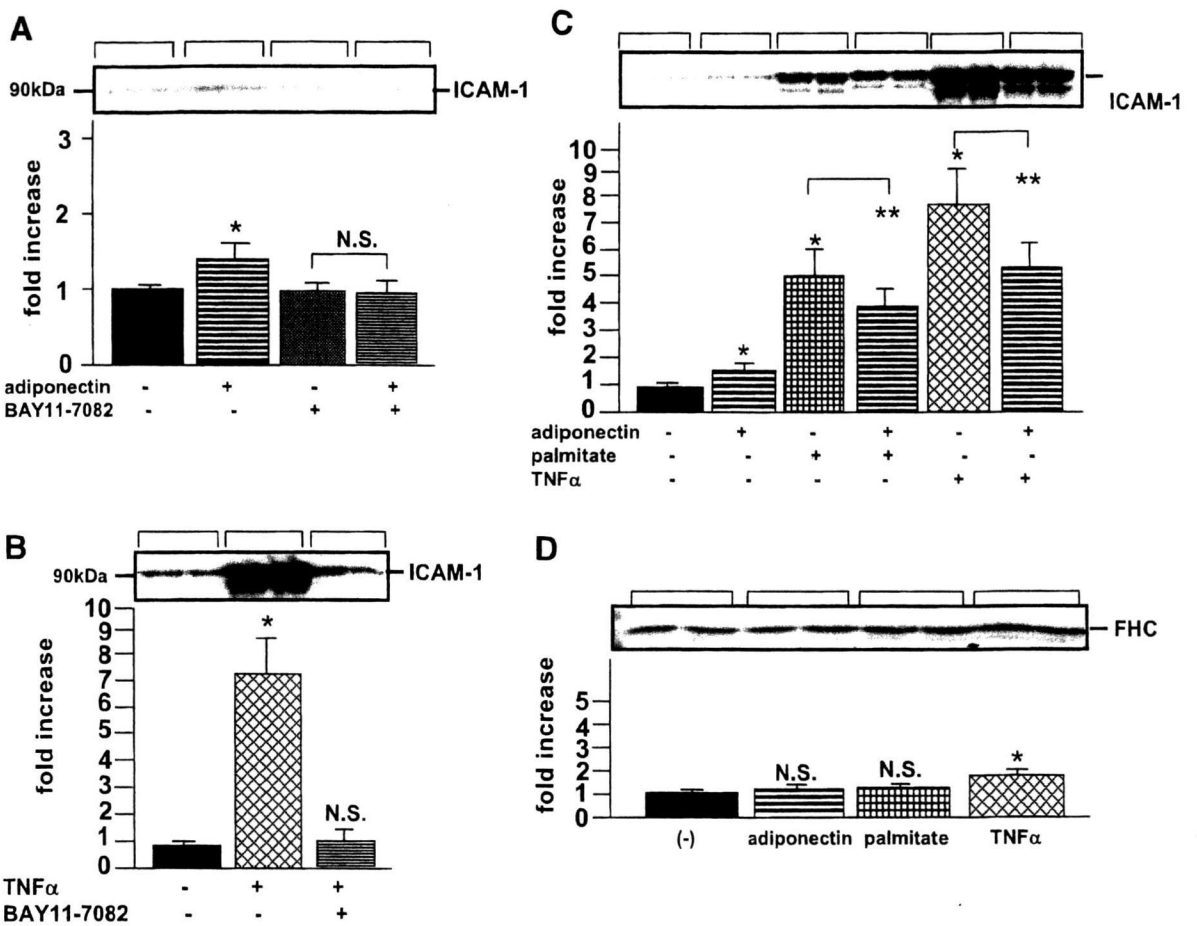

FIG. 4. Effects of recombinant human adiponectin, TNF-α, and free fatty acids on NF-κB–regulated gene expressions in HUVECs. After pretreating HUVECs with or without 100 μmol/l BAY11-7082 for 1 h, 25 μg/ml of adiponectin (*A*) or 10 ng/ml of TNF-α (*B*) were added, followed by incubation for 12 h. After 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

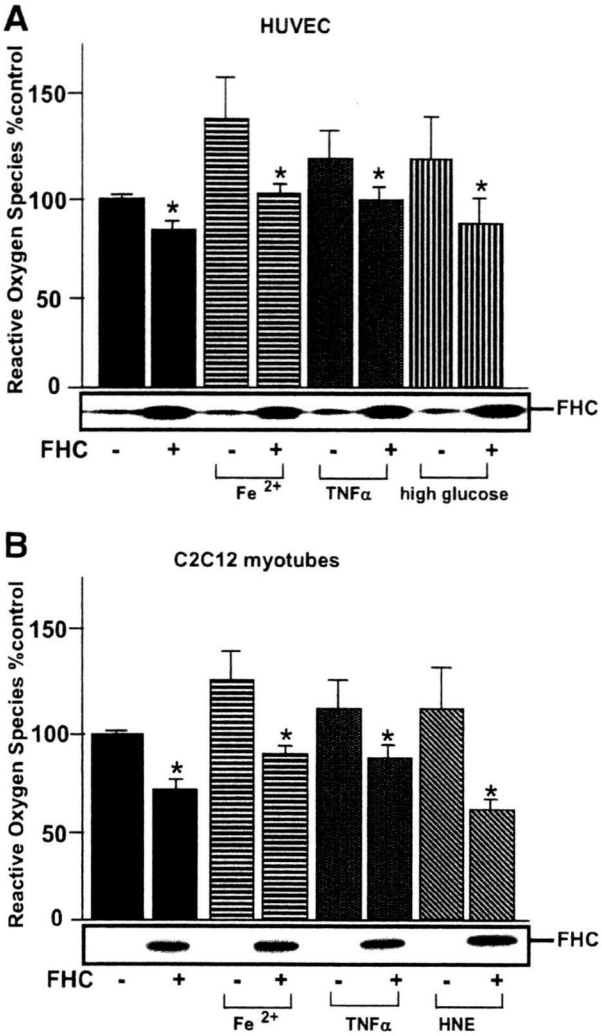

FIG. 5. Effects of recombinantly overexpressed FHC on ROS generation in C2C12 myotubes and HUVECs exposed to oxidative stresses. *A*: HUVECs, transfected with adenovirus overexpressing LacZ or recombinant FHC, were treated with 5.4×10^{-9} mol/l iron (II) sulfate heptahydrate and 25 mmol/l glucose. *B*: 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.

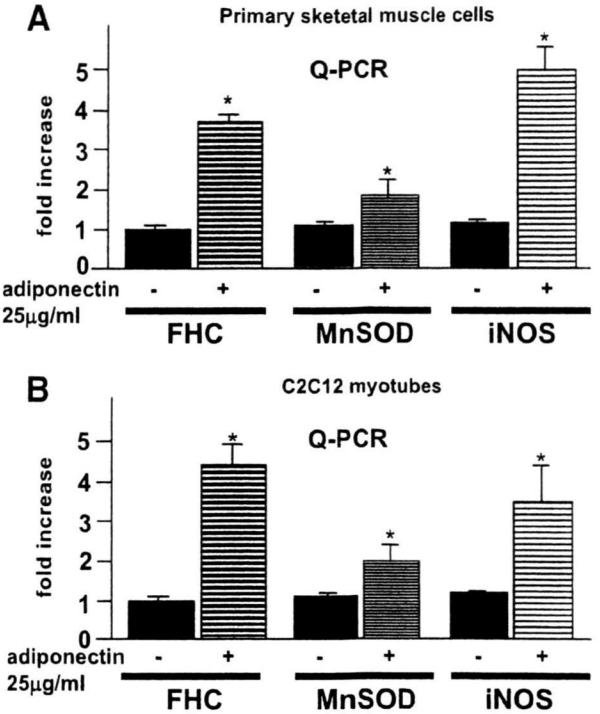

FIG. 6. FHC, MnSOD, and iNOS mRNA levels in primary skeletal muscle cells (*A*) and C2C12 myotubes (*B*) were determined by quantitative PCR using an ABI PRISM model 7000 according to the manufacturer's instructions. Each column shows the mean ± SE obtained from four samples in the presence or absence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

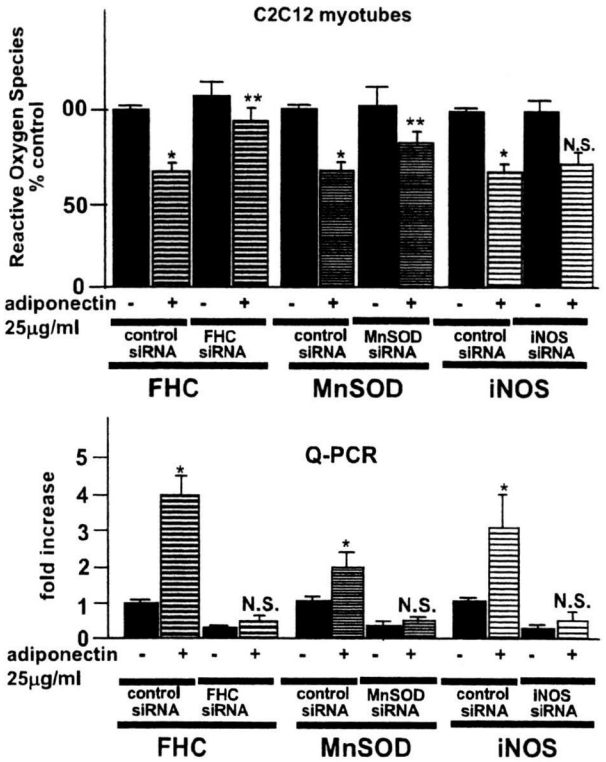

FIG. 7. C2C12 myotubes were transfected with the control nonsilencing siRNA and siRNA against FHC, MnSOD, and iNOS using the transfection reagent. The cells were used for experiments 48 h after siRNA transfection and incubated with or without recombinant adiponectin at 12 h before the experiments. ROS 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

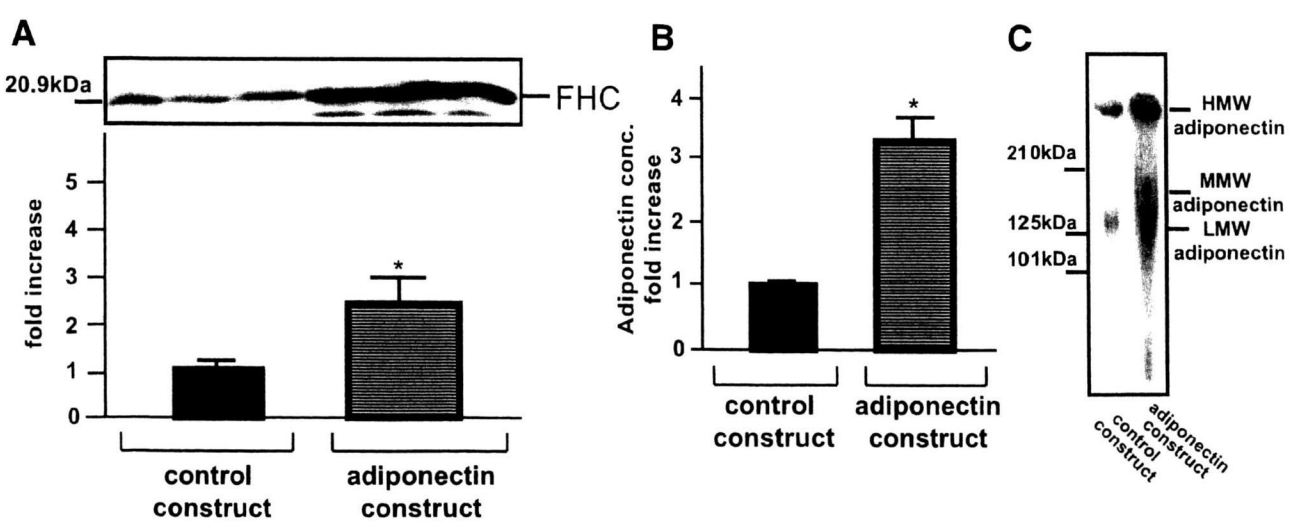

FIG. 8. Effects of adiponectin overexpression on FHC regulation in skeletal muscles. Mice were systemically injected with recombinant adenovirus expressing LacZ (L group) or adiponectin (A group) via the tail vein. Three days after virus infection, we confirmed increased serum adiponectin levels, using 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

This study was supported by Grant-in-Aid for Scientific Research no. 18591001 (to K.I.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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

We thank Y. Asami for help with some of the experiments and are grateful to S. Ohshima and N. Hirosawa for their excellent technical assistance with the flow cytometry and the two-dimensional electrophoresis, respectively.

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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].

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S. Nakanishi et al. (eds.), *Systems Biology*,
DOI:10.1007/978-4-431-87704-2_23, © Springer 2009