ERK5 Activation Inhibits Inflammatory Responses via Peroxisome Proliferator-activated Receptor δ (PPARδ) Stimulation

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Peroxisome proliferator-activated receptors (PPAR) decrease the production of cytokine and inducible nitric-oxide synthase (iNOS) expression, which are associated with aging-related inflammation and insulin resistance. Recently, the involvement of the induction of heme oxygenase-1 (HO-1) in regulating inflammation has been suggested, but the exact mechanisms for reducing inflammation by HO-1 remains unclear. We found that overexpression of HO-1 and [Ru(CO)3Cl2], a carbon monoxide (CO)-releasing compound, increased not only ERK5 kinase activity, but also its transcriptional activity measured by luciferase assay with the transfection of the Gal4-ERK5 reporter gene. This transcriptional activity is required for coactivation of PPARδ by ERK5 in C2C12 cells. [Ru(CO)3Cl2] activated PPARδ transcriptional activity via the MEK5/ERK5 signaling pathway. The inhibition of NF-κB activity by ERK5 activation was reversed by a dominant negative form of PPARδ suggesting that ERK5/PPARδ activation is required for the anti-inflammatory effects of CO and HO-1. Based on these data, we propose a new mechanism by which CO and HO-1 mediate anti-inflammatory effects via activating ERK5/PPARδ, and ERK5 mediates CO and HO-1-induced PPARδ activation via its interaction with PPARδ.

Muscle wasting is a major feature of the cachexia associated with diverse pathologies such as cancer, sepsis, diabetes, and aging (1). Several cytokines have been implicated in the pathogenesis of muscle wasting, most notably TNF-α, a pro-inflammatory cytokine that was originally called “cachectin” (1). In addition, aging-related chronic low grade inflammation by TNF-α plays an important role in insulin resistance (2). It has been proposed that chronic inflammation by TNF-α-mediated NF-κB activation and subsequent inducible nitric-oxide synthase (iNOS) induction relates to muscle wasting and insulin resistance as we will explain below. Cai et al. (3) have shown that activation of NF-κB, through muscle-specific transgenic expression of activated IκB kinase β (MIKK), causes profound muscle wasting that resembles clinical cachexia. In contrast, no overt phenotype was seen upon muscle-specific inhibition of NF-κB through expression of IκB suppressor (MISR), and denervation and tumor-induced muscle loss were substantially reduced and survival rates improved by NF-κB inhibition in MISR mice, which is consistent with a critical role for NF-κB in the pathology of muscle wasting, especially in diabetes and during the process of aging (3). Recent studies suggest the involvement of iNOS in the pathogenesis of insulin resistance (4, 5). First, most inducers of insulin resistance, including obesity (6), free fatty acids (7), hyperglycemia (8, 9), TNF-α, oxidative stress, and endotoxin, increase iNOS expression. Second, iNOS mediates the impaired insulin-stimulated glucose uptake by treatment with TNF-α and lipopolysaccharide in cultured muscle cells (10). iNOS expression is elevated in skeletal muscle of patients with type 2 diabetes (11, 12), and high fat diet-induced diabetic mice (4). Finally, Perreault and Marette (4) showed that the knockout of iNOS specifically from skeletal muscle protects against high fat diet-induced insulin resistance in mice (4).

Heme oxygenases (HO) are the rate-limiting enzymes in the degradation of heme to carbon monoxide (CO), bilirubin, and iron. Three HO isoforms have been identified. The dominant isoform in muscle is HO-1, which is induced by stressful and inflammatory stimuli (13). Interestingly, it has been reported that HO-1 mRNA is lower in patients with type 2 diabetes compared with healthy control subjects (14). In addition, Pilegaard et al. (15) have shown that there is a significant increase of HO-1 expression associated with fatty acid oxidation enzyme expression (UCP-3, PDK4) in response to exercise. Although HO-1 provides protection against proinflammatory cytokines (16), little is known regarding the functional significance of HO-1 in skeletal muscle (15).

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors, which form a subfamily of the nuclear gene family. Three related PPAR isotypes have
been identified to date: PPARα, PPARβ/δ, and PPARγ. The tissue distribution patterns of the PPAR isoforms vary considerably. PPARα is highly expressed in brown adipose tissue, skeletal muscle, liver, heart, and kidney, whereas expressed at low levels in the brain and lung (17). The principal site of expression of PPARγ is the adipose tissue, but PPARγ is also expressed, albeit at lower levels, in many other tissues and cell types (18). PPARδ is found in higher amounts than PPARα and -γ in almost all tissues examined, except adipose tissue (19). Among the most studied role of PPARs is their involvement in inflammatory processes. Numerous studies showed that PPARα and -γ possess anti-inflammatory effects both in vitro and in vivo. Furthermore, specific PPARδ ligands inhibit the expression of proinflammatory cytokines in both macrophages and endothelial cells (20, 21), and ischemia-mediated inflammation in the kidney is enhanced in PPARδ knockout mice (22). However, the anti-inflammatory role of PPARδ in skeletal muscle remains largely unknown.

In addition to their ligand-mediated activation, PPARs activity is regulated by their phosphorylation status. MAP kinase signaling pathways have been implicated in the regulation of nuclear receptor function including PPARγ. A putative MAP kinase site is phosphorylated by ERK1/2 and JNK (23). Phosphorylation significantly inhibits both ligand-independent and ligand-dependent transcriptional activation by PPARγ (23). This repression is mediated by MAP kinase phosphorylation of Ser-82 on PPARγ-1 (24). MAP kinase can regulate PPARα transcriptional activity, but the exact regulatory mechanism remains unclear (25, 26). We found that the association of PPARγ and ERK5 at the hinge-helix 1 region up-regulates PPARγ transcriptional activity by releasing the repressor of SMRT (27). In contrast to PPARα and -γ, the regulation of PPARδ by MAP kinases remains largely unknown.

In the current study, we found that activation of ERK5 increased PPARδ transcriptional activity in C2C12 skeletal muscle cells. PPARδ ligands enhance insulin sensitivity and slow the progression of insulin resistance. Skeletal muscle-specific PPARδ knockout mice have been shown to be prone to insulin resistance (28, 29). We found that a CO releasing compound, [Ru(CO)₃Cl₂]₂, and HO-1 induction significantly slow the progression of insulin resistance. Skeletal muscle-specific PPARδ knockout mice have been shown to be prone to insulin resistance (28, 29). We found that a CO releasing compound, [Ru(CO)₃Cl₂]₂, and HO-1 induction significantly slow the progression of insulin resistance.

Plasmid Construction and Adenovirus Vectors—PPARδ cDNA was a kind gift from Dr. Andrew Billin (GaxoSmithKine). Mouse ERK5 and the constitutively active form of MEK5α (30) (CA-MEK5α) were cloned as described previously (33). Gal4-PPARδ, various deletions of Gal4-PPARδ, and Gal4-ERK5 were created by cloning PCR-amplified DNA fragments corresponding to the different mouse PPARδ or ERK5 regions into the SalI and NotI sites of the pBIND vector. VP16-PPARδ and various deletions of VP16-ERK5 were created by cloning PCR-amplified DNA fragments corresponding to the different PPARδ or ERK5 regions into the pBIND and pACT vectors, respectively. Glutathione S-transferase (GST)-PPARδ was created by cloning PCR-amplified DNA fragments corresponding to the different PPARδ regions into the EcoRI and XhoI sites of the pGEX-KG vector (Amersham Biosciences). The single or double mutations of PPARδ and ERK5 were created with the QuikChange site-directed mutagenesis kit (Stratagene). The ERK5 and PPARδ deletion mutants were created by cloning PCR-amplified DNA fragments corresponding to the different mouse PPARδ or ERK5 regions into SalI and NotI sites of the pBIND vector. All constructs were verified by DNA sequencing. Adenovirus expressing the constitutively active forms of MEK5α (Ad-CA-MEK5α) and HO-1 (Ad-HO-1) were kind gifts from Dr. Jay Yang (Columbia University) (31) and Dr. Raymond F. Regan (Thomas Jefferson University) (32), respectively. Adenovirus containing β-galactosidase (Ad-LacZ) was used as a control virus. Dominant negative MEK1 (DN-MEK1; S218A/T222A) and the constitutively active form of MEK1 (CA-MEK1; S218E/T222E) were described previously (31). Dominant negative MEK4 (DN-MEK4; S220A/T224L) and constitutively active MEK4 (CA-MEK4; S220E/T224D) were kind gifts from Dr. Aubrey Morrison (Beth Israel Deaconess Medical Center).

PathDetect in Vivo Signal Transduction Pathway Reporting System—NF-κB activity was assayed using the PathDetect Signal Transduction Pathway trans-Reporting Systems (Stratagene) as described previously (33). The pRL-TK Renilla luciferase vector was used for normalization of transfection. Differentiated cells were co-transfected with pNF-κB-Luc reporter plasmid and pRL-TK with other plasmids as indicated in the figures.

Mammalian One- or Two-hybrid Analysis and Transfection of C2C12 Cells—C2C12 cells were plated in 12-well plates at 5 × 10⁴ cells/well and 24 h later incubated in Dulbecco’s modified Eagle’s medium supplemented with 5% horse serum for 3 days to differentiate cells into skeletal muscle cells. For the mammalian two-hybrid assay, cells were transfected in a OptiMEM (Invitrogen) with Lipofectamine mixture containing the pG5-luc vector and various pBIND and pACT plasmids (Promega). After 4 h, cells were washed and fresh Dulbecco’s modified Eagle’s medium supplemented with 5% horse serum was added. The pG5-luc vector contains five Gal4 binding sites upstream of a minimal TATA box that, in turn, is upstream of the firefly luciferase gene. pBIND and pACT contain Gal4 and VP16, respectively, and were fused with PPARδ and ERK5, as experimental procedures

Cell Culture—The C2C12 mouse myoblast line was maintained with Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Differentiation was induced in C2C12 cells grown in 12-well dishes at about 90% confluence by replacing medium with Dulbecco’s modified Eagle’s medium containing 5% horse serum for 3 days.
vectors for the indicated times. Transfections were performed in triplicate, and each experiment was repeated at least two times.

*In Vitro Phosphorylation of PPARδ by Activated ERK5*—GST-PPARδ protein was expressed in *Escherichia coli* and purified using glutathione-Sepharose 4B as described by the manufacturer (GE Healthcare). ERK5 activity was measured as previously described (27). To determine whether PPARs can be phosphorylated by activated ERK5, we performed an ERK5 *in vitro* kinase assay with GST-PPARδ as the substrate.

**Immunoprecipitation and Western Blot Analysis**—The cells were washed with phosphate-buffered saline and harvested in 0.5 ml of lysis buffer as described previously (37). Immunoprecipitation was performed as described previously with anti-ERK5 antibody (27) or anti-PPARδ antibody (Santa Cruz). Western blot analysis was performed as previously described (27). In brief, the blots were incubated for 4 h at room temperature with the anti-ERK5 (1), iNOS (Cayman), tubulin (Sigma), p38, ERK1, JNK1/2 (Cell Signaling), HO-1, PPARα, PPARγ, PPARδ, hemagglutinin (Santa Cruz), and anti-phospho-ERK5, ERK1/2, and JNK1/2 (Cell signaling), or Xpress (Invitrogen) antibody, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham Biosciences).

**Materials**—GW610742 compound was a kind gift from Dr. Andrew Billin (GaxoSmithKine). [Ru(CO)3Cl2]2 from Sigma.

**Figure 1.** HO-1 induction and [Ru(CO)3Cl2]2-inhibited TNF-α-mediated NF-κB activation and iNOS expression in C2C12 cells. A, C2C12 cells were transfected with pFRLuc plasmid and pNF-κB luc vector, pBind-ERK5 and pCMV tag, or pCMV-CA-MEK5 and then normalized to cotransfected luciferase activity as described under “Experimental Procedures.” Results are the mean ± S.D. of three independent experiments. **, p < 0.01. Right panel, C2C12 cells were transfected with pFR-Luc plasmid and pIBoh-Luc vector with or without Ad-LacZ or Ad-HO-1 transduction. To control transfection efficiency, pRL-TK was transfected as a luciferase control reporter vector. After 12 h of transfection, C2C12 were treated with vehicle or TNF-α stimulation (left panel). After 12 h of TNF-α stimulation, luciferase NF-κB transcriptional activity was assayed using the dual-luciferase reporter assay system, and luciferase luminescence was counted in a Luminometer and normalized to transfection efficiency. B, C2C12 cells were transfected with pFRLuc plasmid and pNF-κB luc vector as described above, and after 12 h of transfection, cells were treated by vehicle or TNF-α stimulation. After 12 h of TNF-α stimulation, luciferase NF-κB transcriptional activity was assayed using the dual-luciferase reporter assay system as described above. C, C2C12 cells were transduced with or without Ad-LacZ or Ad-HO-1. After 16 h of transduction, C2C12 were treated by vehicle or TNF-α stimulation. After 6 h of TNF-α stimulation, iNOS, HO-1, and tubulin expression were determined by Western blot analysis. C, C2C12 cells were treated with or without [Ru(CO)3Cl2]2, as indicated. Amounts flowed by vehicle or TNF-α stimulation. After 6 h of TNF-α stimulation, iNOS and tubulin expression were determined by Western blot analysis. IB, Immunoblot. *, p < 0.05.

indicated. Because pBIND also contains the *Renilla* luciferase gene, the expression and transfection efficiencies were normalized with the *Renilla* luciferase activity. Cells were collected 36 h after transfection except as indicated, and the luciferase activity was assayed with the dual luciferase kit (Promega) using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). In the case of the mammalian one-hybrid analysis for the ERK5 transcriptional activity, differentiated cells were transfected with pG5-luc vector, pBind-ERK5 and pCMV tag, or pCMV-CA-MEK5α and then exposed to [Ru(CO)3Cl2]2, GW610742, or adenoviral
ERK5/PPARδ Activation in Skeletal Muscle

FIGURE 2. HO-1 induction and [Ru(CO)3Cl2]2 activate ERK5 kinase and transcriptional activity. A, C2C12 cells were transduced with Ad-HO-1 and after a 24-h transfection, ERK5 kinase activation and induction of HO-1 were examined by Western blot analysis with anti-phospho-ERK5 and anti-HO-1 antibody. No significant differences were observed in total ERK5 (second panel from top). B and C, Gal4-ERK5 transcriptional activity was detected. C2C12 cells were co-transfected with Gal4-ERK5 and Gal4-dependent (Gal4-luc) reporter constructs with Ad-LacZ or Ad-HO-1 transduction (B). After 24 h of transfection, luciferase activity was measured as described previously (27). E, C2C12 cells were co-transfected with Gal4-ERK5 and Gal4-dependent (Gal4-luc) reporter constructs. After a 12-h transfection (E) C2C12 cells were incubated with [Ru(CO)3Cl2]2 at the indicated doses for 16 h and luciferase activity was measured. F, C2C12 cells were incubated with insulin growth factor 1 (IGF-1) (50 ng/ml), basic fibroblast growth factor (bFGF) (25 ng/ml), 5% horse serum, and 10% fetal bovine serum (FBS) for 16 h and luciferase activity was measured. C and D, C2C12 were treated with or without [Ru(CO)3Cl2]2 (30 μM) and a time course of activation of ERK5 was examined by Western blot analysis with anti-phospho-ERK5. No significant differences were observed in total ERK5 (C, bottom). D, ERK5 kinase activity was increased by [Ru(CO)3Cl2]2 dose-dependently. DMSO, dimethyl sulfoxide. *p < 0.05; **p < 0.01.

ies against ERK5 (Cell Signaling), ERK1/2 (Cell Signaling), or tubulin (Sigma).

Statistical Analysis—Data are reported as mean ± S.D. Statistical analysis was performed with the StatView 4.0 package (ABACUS Concepts, Berkeley, CA). Differences were analyzed with one-way or two-way repeated measure analysis of variance as appropriate, followed by Scheffe’s correction for multiple comparisons. p values less than 0.05 are indicated by a single asterisk and less than 0.01 by double asterisks.

RESULTS

HO-1 Induction and [Ru(CO)3Cl2]2 Attenuated Proinflammatory Responses to TNF-α in Skeletal Muscle Cells—To determine the effect of HO-1 and a CO releasing compound, [Ru(CO)3Cl2]2, on proinflammatory responses in skeletal muscle cells, we first examined these effects on TNF-α-mediated NF-κB activation. As shown in Fig. 1A, both HO-1 induction and [Ru(CO)3Cl2]2 could inhibit TNF-α-mediated NF-κB activation. Because iNOS expression in skeletal muscle has a significant role in inflammatory responses and subsequent insulin resistance especially in the elderly (4, 5), we investigated whether induction of HO-1 and [Ru(CO)3Cl2]2 can inhibit TNF-α-mediated iNOS expression. We found that the induction of iNOS in response to TNF-α was significantly decreased by HO-1 induction (Fig. 1B) and CO (Fig. 1C). These data suggest an anti-inflammatory role for HO-1 induction as well as subsequent CO release in skeletal muscle.

HO-1 Induction and [Ru(CO)3Cl2]2 Activate ERK5 Kinase and Transcriptional Activity—To determine the effect of HO-1 and the subsequent HO-1 product CO on ERK5 activation, we first transduced adenovirus containing HO-1 (Ad-HO-1) to C2C12 cells and confirmed that ERK5 kinase activation was increased (Fig. 2A). Next we treated the C2C12 skeletal muscle cells with [Ru(CO)3Cl2]2. We found that [Ru(CO)3Cl2]2 significantly increased ERK5 kinase activation from 5 to 15 min after [Ru(CO)3Cl2]2 stimulation, and ERK5 phosphorylation, which represents kinase activity, was dose-dependently increased by [Ru(CO)3Cl2]2 (Fig. 2, C and D).

Previously we reported using the Gal4-ERK5 construct to detect ERK5 transcriptional activity (27). We utilized this construct and detected ERK5 transcriptional activity induced by Ad-HO-1 and [Ru(CO)3Cl2]2. As shown in Fig. 2, B and E, we found that both transduction of Ad-HO-1 and [Ru(CO)3Cl2]2 significantly increased ERK5 transcriptional activity. We also compared the effect of [Ru(CO)3Cl2]2 with other well known ERK5 activators such as serum or insulin growth factor 1. [Ru(CO)3Cl2]2 induced comparable ERK5 transcriptional activity as the other physiological activators (Fig. 2F), supporting the possible physiological role of [Ru(CO)3Cl2]2 and HO-1-mediated ERK5 transcriptional activation.

Activation of ERK5 Increased PPARδ Transcriptional Activity and Inhibited Inflammatory Responses in Response to TNF-α—Previously we reported that PPARδ transcriptional activity was increased by ERK5 in endothelial cells (27). Therefore, in this study we investigated whether ERK5 can increase PPARδ activity in skeletal muscle because of the significant expression of PPARδ in skeletal muscle (29). We co-expressed PPARδ and
ERK5/PPAR\(\delta\) Activation in Skeletal Muscle

![Figure 3](image)

**FIGURE 3.** ERK5 activation increased PPAR\(\delta\) transcriptional activity, and PPAR\(\delta\) ligand inhibited TNF-\(\alpha\)-mediated NF-\(\kappa\)B activation and iNOS expression. (A) MEK5\(\alpha\)-ERK5 activation induced PPAR\(\delta\) transcriptional activity. PPAR\(\delta\) transcriptional activity was measured by transfection of full-length PPAR\(\delta\) and the (PPRE)3-Tk-luciferase reporter construct in C2C12 cells. PPAR\(\delta\) transcriptional activity was determined with the transfection of CA-MEK5\(\alpha\) empty vector with vehicle or GW610742 at the indicated doses. (B) and (C), PPAR\(\delta\) ligand, GW610742, significantly inhibited NF-\(\kappa\)B-mediated NF-\(\kappa\)B activation and iNOS expression. (B), after 24 h of NF-\(\kappa\)B reporter gene transfection, C2C12 cells were incubated with GW610742 at the indicated doses. After 1 h of GW610742 treatment, cells were stimulated with TNF-\(\alpha\) or vehicle for 16 h and NF-\(\kappa\)B activity was determined by luciferase activity as described in the legend to Fig. 1. C, C2C12 cells were incubated with vehicle or GW610742 (100 nM) followed by TNF-\(\alpha\) or vehicle stimulation for 6 h. Western blot analysis was performed with anti-iNOS (top) and actin (middle) antibody. Bottom, densitometric analysis of iNOS expression. Results were normalized by arbitrarily setting the densitometry of vehicle treated cells to 1.0 (shown is mean + S.D., n = 3); *, \(p < 0.05\); **, \(p < 0.01\).

Because PPAR\(\delta\) activation has been reported to be anti-inflammatory (21), we first studied the role of PPAR\(\delta\) activation on TNF-\(\alpha\)-mediated inflammatory responses. We found that PPAR\(\delta\) ligand GW610742 significantly inhibited TNF-\(\alpha\)-mediated NF-\(\kappa\)B activity (Fig. 3B) and subsequent iNOS induction (Fig. 3C). Next, because we found that forced activation of ERK5 increased PPAR\(\delta\) transcriptional activity, we investigated whether activation of ERK5 can prevent TNF-\(\alpha\)-mediated inflammatory responses. We transduced adenovirus CA-MEK5\(\alpha\) (Ad-CA-MEK5\(\alpha\)) and transfected NF-\(\kappa\)B reporter gene, and after 24 h of transfection C2C12 cells were incubated with TNF-\(\alpha\) (20 ng/ml) for 16 h and NF-\(\kappa\)B activity was detected by luciferase activity. As shown in Fig. 4A, activation of ERK5 significantly decreased TNF-\(\alpha\)-mediated NF-\(\kappa\)B activity. To determine the unique role of ERK5 activation as an inflammation inhibitor, we also investigated the role of MEK1-ERK1/2 and MEK4-JNK1/2 pathways on TNF-\(\alpha\)-mediated NF-\(\kappa\)B activity using the constitutively active forms of MEK1 (CA-MEK1) and MEK4 (CA-MEK4). We confirmed that transfection of these molecules could increase Elk-1 and c-Jun transcriptional activity, respectively (data not shown). As shown in Fig. 4, B and C, we found that both CA-MEK1 and CA-MEK4 have no effect on TNF-\(\alpha\)-mediated NF-\(\kappa\)B activation in C2C12 cells, suggesting the unique anti-inflammatory effect of MEK5-ERK5 activation on TNF-\(\alpha\)-mediated NF-\(\kappa\)B activation.

Furthermore, we found that transduction of Ad-CA-MEK5\(\alpha\) significantly inhibited TNF-\(\alpha\)-mediated iNOS induction (Fig. 4, D and E). Taken together, these data suggest that both PPAR\(\delta\) and ERK5 could inhibit the inflammatory response induced by TNF-\(\alpha\).

**ERK5 and PPAR\(\delta\) Activation Are Critical for the Inhibitory Effect of CO on TNF-\(\alpha\)-mediated NF-\(\kappa\)B Activation**—Because we found that HO-1 induction and [Ru(CO)\(_3\)Cl\(_2\)]\(_2\) increased ERK5 activation and specific activation of ERK5 could increase PPAR\(\delta\) transcriptional activity, we investigated whether [Ru(CO)\(_3\)Cl\(_2\)]\(_2\) could increase PPAR\(\delta\) transcriptional activity through ERK5 activation. We transfected a dominant negative form of MEK5\(\alpha\) (MEK5\(\alpha\)-DN) and MEK4-JNK1/2 pathways on TNF-\(\alpha\)-mediated NF-\(\kappa\)B activity. We confirmed that MEK1-ERK1/2 and MEK4-JNK1/2 pathways on TNF-\(\alpha\)-mediated NF-\(\kappa\)B activity using the constitutively active forms of MEK1 (CA-MEK1) and MEK4 (CA-MEK4). We confirmed that transfection of these molecules could increase Elk-1 and c-Jun transcriptional activity, respectively (data not shown). As shown in Fig. 5A, we found that [Ru(CO)\(_3\)Cl\(_2\)]\(_2\) increased PPAR\(\delta\) activity and the co-expression of DN-MEK5\(\beta\) significantly decreased [Ru(CO)\(_3\)Cl\(_2\)]\(_2\)-mediated PPAR\(\delta\) activity. Note, we did not observe any inhibitory effect of DN-MEK5\(\beta\) on the PPAR\(\delta\) ligand-mediated PPAR\(\delta\) activity (data not shown). We confirmed similar inhibition of [Ru(CO)\(_3\)Cl\(_2\)]\(_2\)-mediated PPAR\(\delta\) activity by transfection of DN-ERK5 (Fig. 5A), suggesting the critical role of ERK5 activation on CO-, but not PPAR\(\delta\) ligand, mediated PPAR\(\delta\) activation. Because of the limitation by the low transfection efficiency of the reporter gene in C2C12 cells, our reporter gene assay showed marginal changes. However, the amount of
**PPARγ/H9254 activation by [Ru(CO)3Cl2]2 (60 μM) is similar to that induced by the PPARγ activator GW610742 (30 μM) (Figs. 3A and 5). PPARγ has a significant effect on TNF-α-mediated inflammatory responses as we and others reported (20, 21, 34). Hence, we anticipate that PPARγ activation by [Ru(CO)3Cl2]2 has a significant pathological effect in skeletal muscle.**

To confirm the role of ERK5 activation on [Ru(CO)3Cl2]2-mediated PPARγ transcriptional activation, we utilized ERK5 siRNA to knockdown its expression. In C2C12 cells the transfection of ERK5 but not control siRNA significantly decreased ERK5 expression (Fig. 5B). As shown in Fig. 5C, we found that [Ru(CO)3Cl2]2-mediated PPARγ activity was significantly impaired by deletion of ERK5 expression in C2C12 cells. These data also support the critical role of ERK5 in [Ru(CO)3Cl2]2-mediated PPARγ transcriptional activity. To determine the possible involvement of MEK1 and MEK4 activation on [Ru(CO)3Cl2]2-mediated PPARγ activation, we used the DN-MEK1 and DN-MEK4 showed no effect on PPARγ activation, also suggesting the unique role of MEK5/ERK5 on [Ru(CO)3Cl2]2-mediated PPARγ activation.

To show the physiological role for ERK5/PPARγ activation by [Ru(CO)3Cl2]2, we studied the role of ERK5 activation in the inhibitory effect of [Ru(CO)3Cl2]2 on TNF-α-mediated NF-κB activation. Because DN-ERK5 significantly inhibited [Ru(CO)3Cl2]2 on TNF-α-mediated NF-κB activation. As shown in Fig. 6A, DN-ERK5 significantly inhibited the ability of [Ru(CO)3Cl2]2 to decrease the TNF-α-mediated NF-κB activation. These data suggest that the inhibitory effect of [Ru(CO)3Cl2]2 on NF-κB activation, is at least partially, due to the activation of ERK5.

Furthermore, to investigate the involvement of PPARγ activation of ERK5-mediated inhibition of NF-κB activation, we utilized a dominant negative form of PPARγ (DN-PPARγ, PPARγE411P). As shown in Fig. 6B, CA-MEK5α (lanes 8–10), and PPARγ ligand, GW610742 (lanes 3–5), significantly inhibited TNF-α-mediated NF-κB activation. Transfection of DN-
PPARδ significantly decreased the inhibitory effect of ERK5 activation on NF-κB activation (Fig. 6B, lanes 5, 10, and 11), suggesting that the inhibitory effect of ERK5 activation is due to its activation of PPARδ.

ERK5 Kinase Did Not Phosphorylate PPARδ in Vitro, but Could Associate with PPARδ—Because activation of ERK5 regulates PPARδ activity, we asked whether ERK5 could phosphorylate PPARδ in vitro. We cotransfected CA-MEK5α and Xpress-tagged ERK5 in COS7 cells to activate ERK5 constitutively. Activated ERK5 was immunoprecipitated with an anti-ERK5 antibody, and in vitro kinase assay was performed with GST and GST full-length PPARδ as substrates. As shown in Fig. 7A, transfection of CA-MEK5α activated ERK5 kinase, as shown by ERK5 autophosphorylation (Fig. 7A, bottom). However, ERK5 did not phosphorylate PPARδ (Fig. 7A, top).

Previously, we reported that ERK5 regulates PPARγ transcriptional activity via the association between ERK5 and PPARγ (27). Therefore, to investigate the potential interaction between ERK5 and PPARδ, we analyzed their interaction using co-immunoprecipitation. Because PPARδ is a nuclear receptor and ERK5 needs to be activated for its nuclear translocation (35), we stimulated the cells with 10% serum for 30 min and immunoprecipitated the cells with an anti-PPARδ antibody or rabbit immunoglobulin G (IgG) as a control. We found that endogenous PPARδ co-immunoprecipitated with endogenous ERK5 in skeletal muscle cells, but control IgG did not (Fig. 7B).

To confirm the binding site of ERK5 with PPARδ, we utilized a mammalian two-hybrid assay. A plasmid expressing the GAL4-DBD and the PPARδ (full-length) was constructed by inserting PPARδ into p535-PPARδ and ERK5 needs to be activated for its nuclear translocation (35), we stimulated the cells with 10% serum for 30 min and immunoprecipitated the cells with an anti-PPARδ antibody or rabbit immunoglobulin G (IgG) as a control. We found that endogenous PPARδ co-immunoprecipitated with endogenous ERK5 in skeletal muscle cells, but control IgG did not (Fig. 7B).

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suggesting the involvement of ERK5 kinase activation on ERK5/PPARδ association.

We next generated several deletion mutants of ERK5 to define the domains required for PPARδ association. ERK5 deletion mutants were cloned into the VP16 active domain plasmid, and the interactions with Gal4-PPARδ were determined in a two-hybrid mammalian assay. As shown in Fig. 8A, the deletion mutant ERK5 (aa 78–806 and 412–806), but not ERK5 (aa 571–806 and 1–418), associated with PPARδ, suggesting that 412–570 contains the ERK5 binding domain of PPARδ. To rule out the possibility of another binding site in the COOH-terminal region, we also generated the ERK5 (aa 684–806) containing the ERK5 COOH-terminal tail (aa 684–806) alone could not induce PPARδ activity, suggesting a critical role for the arginine-rich region of ERK5 (aa 412–577) as a binding site of ERK5 for PPARδ, which is similar to PPARγ as we previously reported (27). Of note, although ERK5 aa 412–570 contains a nuclear localizing signal (aa 505–539 (36)), the nuclear localizing signal in VP-16 compensates this defect and should not affect ERK5/PPARδ binding due to its localization (27).

Next, we investigated the binding site of PPARδ on ERK5. As shown in Fig. 8B, we found that the deletion mutant of hinge-helix 1 region of PPARδ (aa 270–295) could associate with ERK5, which is different from PPARγ, suggesting that ERK5 may associate with another domain besides the hinge-helix 1 region of PPARδ.

The Binding Site of ERK5 to PPARδ Is Required for ERK5-mediated PPARδ Activation—Previously, we reported that the ERK5 COOH-terminal tail (aa 684–806) had very high transcriptional activity even without CA-MEK5α transfection (27). Therefore, to determine the role of ERK5/PPARδ association on PPARδ activation, we generated several VP-16-fused COOH-terminal deletion mutants containing the ERK5 aa 684–806 site. As shown in Fig. 8C, the entire COOH-terminal ERK5 (aa 412–806), which contains both the PPARδ binding site and aa 684–806 transactivation domain, increased PPARδ activation. However, the transactivation domain at the COOH-terminal tail of ERK5 (aa 684–806) alone could not induce PPARδ activity, suggesting a critical role for the arginine-rich region of ERK5 (aa 412–577) as a binding site of ERK5 with PPARδ.

**DISCUSSION**

In the present study we investigated whether HO-1 and CO can inhibit TNF-α-mediated inflammatory responses in skeletal muscle, which are considered to be events essential for insulin resistance in the elderly. We found that CO and induction of HO-1 inhibited TNF-α-mediated inflammatory responses. To determine the mechanism of anti-inflammatory effects of CO
induction and a CO-releasing compound, [Ru(CO)3Cl2]2, activated ERK5/PPARδ. Therefore, we proposed a novel anti-inflammatory mechanism for HO-1 mediated by CO-induced ERK5 and PPARδ activation.

There is increasing evidence that CO mediates potent anti-inflammatory effects in various cell types (16, 43, 44). Choi and colleagues (45) reported the critical role of the MKK3/p38 kinase pathway in mediating the anti-inflammatory and anti-apoptotic effects of CO. Although the contribution of MAPK pathways including p38 and ERK1/2 has been reported for the biological effects attributed to CO including anti-inflammatory and anti-apoptotic effects (43–45), the anti-inflammatory mechanisms of ERK1/2 and p38 are not clear (45). Especially, we did not find any inhibitory effect of DN-MEK1 or DN-MEK4 on CO-mediated PPARδ transcriptional activity (Fig. 5, D and E). In addition, we found that both CA-MEK1 and CA-MEK4 could not inhibit TNF-α-mediated NF-κB activation (Fig. 4, B and C), suggesting that ERK1/2 and JNK1/2 may not be major regulators of PPARδ activation and subsequent anti-inflammatory effects induced by CO.

In this study we focused on PPARδ, because PPARδ is one of the major isoforms of PPARs in skeletal muscle (29, 46). We previously reported that ERK5 showed anti-inflammatory effects by stimulating PPARγ transcriptional activity in endothelial cells (27). Here we reported that activation of ERK5 increases PPARδ activation via ERK5/PPARδ association, which is similar to the mechanism of PPARγ activation by ERK5.

ERK5 kinase is a very unique kinase, which possesses both kinase and transcriptional activity (27, 47). The inactive NH2-terminal ERK5 kinase domain acts as a negative regulator of its COOH-terminal region. Kasler et al. (47) reported that the COOH-terminal region of ERK5 contained a MEF2-interacting domain and also a potent transcriptional activation domain. We found that the middle (arginine-rich) region of ERK5, but not the COOH-terminal tail of ERK5, was associated with endogenous PPARδ (Fig. 6). We and other groups (35, 36) have found that kinase activation

and HO-1 induction, we examined MAP kinase activation by [Ru(CO)3Cl2]2 and HO-1 induction, and found that ERK5 can be activated by both [Ru(CO)3Cl2]2 and HO-1. Because we previously reported that the regulation of PPARγ by ERK5 kinase and PPARδ is the major isoform in the skeletal muscle, we determined whether ERK5 can regulate PPARδ activity. Activation of ERK5 increased PPARδ transcriptional activity, and [Ru(CO)3Cl2]2-mediated PPARδ transcriptional activity was inhibited by DN-ERK5. In addition, the inhibition of NF-κB by forced ERK5 activation was reversed by the DN-PPARδ supports the idea that ERK5/PPARδ activation is required for an anti-inflammatory effect of CO and HO-1. Finally, we found that ERK5/PPARδ association is critical for ERK5-mediated PPARδ activation. Based on these data, we propose a new mechanism by which CO and HO-1 mediates the anti-inflammatory effect via activating ERK5/PPARδ.

The cytoprotective role of HO-1 has been observed recently in many kinds of cells including skeletal muscle (37), cardiomyocytes (38), hepatocytes (39, 40), as well as in vascular endothelial cells (16, 41). Several mechanisms could be responsible for the cytoprotective action of the HO-1. First, HO-1 can be an important intracellular antioxidant by degrading the pro-oxidant heme and generate biliverdin and bilirubin, two effective free radical scavengers. The next possible mechanism is down-regulation of iNOS expression by degradation of heme, as an essential cofactor for iNOS protein assembly and activity. Finally, CO, the other product of HO-1, has a protective role and inhibits NO synthase activity (42) or JNK activity and suppresses pro-inflammatory cytokine production (43). In this study, we found that both HO-1 activation and a CO-releasing compound, [Ru(CO)3Cl2]2, activated ERK5/PPARδ. Therefore, we proposed a novel anti-inflammatory mechanism for HO-1 mediated by CO-induced ERK5 and PPARδ activation.

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of ERK5 initiated the nuclear translocation of ERK5. Therefore, both disruption of the inhibitory effect of the NH2-terminal region of ERK5 and the nuclear translocation of ERK5, which are induced by ERK5 activation, may be required to fully activate PPARγ1. In this study we found the critical role of ERK5 and PPARδ association on ERK5-mediated PPARδ activation and ERK5 kinase activation is necessary for full ERK5/PPARδ association and activation of PPARδ. However, although the hinge-helix region of PPARδ and PPARγ share 48% homology in amino acid sequence, we could not detect ERK5/PPARδ interaction via the hinge-helix 1 region of PPARδ. In addition, although the ERK5 (aa 78–806) deletion mutant possesses no kinase activation like DN-ERK5 (36), ERK5 (aa 78–806) could associate with PPARδ (Fig. 8A), which is different from PPARγ, suggesting that the ERK5 (aa 1–78) region may have an inhibitory effect on ERK5/PPARδ interaction. These data also suggest the unique regulatory mechanism of ERK5 on PPARδ activation, which is different from PPARγ activation. Further studies are required to clarify the role of the PPARδ binding site with ERK5.

Recently, two different laboratories (28, 29) reported the phenotype of skeletal muscle-specific PPARδ overexpressed mice. Both showed significantly increased fatty acid oxidation enzyme expression in skeletal muscle, and particularly Wang et al. (48) found that high fat diet-induced insulin resistance was improved in PPARδ transgenic mice. Furthermore, Wang et al. (48) have reported that systemic PPARδ-deficient mice challenged with a high fat diet showed reduced energy uncoupling and were prone to obesity. These data suggest a critical role for PPARδ in regulating insulin sensitivity, probably by increasing FAO enzyme expression and activity, particularly in skeletal muscle. It is also possible that the anti-inflammatory effect of PPARδ may involve increasing insulin sensitivity, because aging-related chronic low grade inflammation plays an important role in insulin resistance (2), and it is well known that chronic iNOS induction causes muscle insulin resistance (4). Further investigation is required to determine the role of HO-1 and ERK5 reduction in the aging process.

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