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Nitric oxide dysregulates adipocytokine expression in 3T3-L1 adipocytes ☆

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Abstract

Obesity is associated with infiltration of macrophages into adipose tissue, and macrophages are an important source of nitric oxide (NO). Dysregulated production of fat-derived secretory factor, adipocytokine, leads to obesity-linked metabolic disorders. However, it has not been fully determined whether NO might have direct effects on adipocytokine expressions. Here, we show that NO donor treatment downregulated gene expression and secretion of adiponectin, and upregulated mRNA levels of PAI-1 and IL-6. NO donor reduced promoter activity of adiponectin through PPARγ responsive element. Moreover, NO donor activated JNK and NF-κB pathways, and inhibitors of these pathways rescued NO-mediated upregulation of PAI-1 and IL-6. Analysis of adipose tissue of high-fat-fed obese mice showed upregulation of PAI-1 and IL-6 expression, increased synthesis of NO, and downregulation of adiponectin. Our results suggest that increased NO synthesis might be partly responsible for dysregulation of adipocytokines in adipose tissue.

Keywords: Obesity; Nitric oxide; Adiponectin; PAI-1; IL-6

Recent investigations have revealed that white adipose tissue (WAT) produces bioactive molecules called adipocytokine, including leptin, adiponectin, tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor type-1 (PAI-1), and IL-6 [1,2]. Furthermore, dysregulation of adipocytokines is linked to obesity-associated metabolic diseases [1,2]. Adiponectin is an adipocytokine with common characteristics to both C1q family members and TNF-ligand family members [3]. Adiponectin improves glucose and lipid metabolism [4], and exhibits anti-inflammatory properties [5]. PAI-1 is an inhibitor of tissue-type plasminogen activator and urokinase-type plasminogen activator [6], and is involved in many processes, such as fibrinolysis, monocyte migration [7], and angiogenesis [8]. PAI-1 is released from adipose tissue [9], and influences visceral fat accumulation and the associated inflammatory amplification [10]. IL-6 is a proinflammatory cytokine secreted from adipocytes [11], and has a significant role in the inflammatory process in obese adipose tissue [12].

Nitric oxide (NO) is a free radical molecule known to play a significant role in the pathogenesis of inflammation [13]. NO is synthesized from L-arginine by three isoforms of NO synthase (NOS): neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) [14]. Plasma levels of NO metabolites correlate with body mass index and body fat mass [15], and high mRNA levels of eNOS and iNOS have been identified in WAT of obese women [16]. In obese mice and Zucker diabetic fatty rats, overexpression of iNOS has been observed in the liver, skeletal muscle, and WAT [17,18]. Moreover, plasma nitrite levels are elevated modestly in diabetic patients [19].

There is ample evidence to suggest the involvement of NO in the development of insulin resistance in obesity models. iNOS-deficient mice are protected from high-fat

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diet-induced insulin resistance [17], and chronic treatment of diabetic db/db mice with *N*6-(1-iminoethyl)-lysine hydrochloride, a specific iNOS inhibitor, reversed fasting hyperglycemia with concomitant reduction of hyperinsulinemia and improved insulin sensitivity [18]. Unno et al. reported that NO mediates downregulation of leptin in 3T3-L1 adipocytes [20]. However, effects of NO on other adipocytokines remain to be elucidated. Here, we investigated the impact of NO on adiponectin, PAI-1 and IL-6 expressions.

Materials and methods

Materials. Anti-c-Jun N-terminal kinase (JNK) polyclonal antibody (pAb), anti-phosphorylated (phospho)-JNK pAb, anti-p38 mitogen-activated protein kinase (MAPK) pAb, anti-phospho-p38 MAPK pAb, and anti-inhibitor-of-κB (IκB) pAb were purchased from Cell Signaling Technology (Beverly, MA). Anti-adiponectin pAb was from Affinity BioReagents Inc. (Golden, CO). Anti-peroxisome proliferator-activated receptor-γ (PPARγ) pAb was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-β-actin monoclonal Ab was from Sigma (St. Louis, MO). JNK inhibitor (SP600125), IκB kinase (IKK) inhibitor (BAY 117082), and guanylate cyclase inhibitor (ODQ; 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), were from Calbiochem (San Diego, CA). *N*-Acetyl cysteine (NAC), lipopolysaccharide (LPS), and hypoxanthine (HX) were from Sigma. Xanthine oxidase (XO) was from Roche (Indianapolis, IN).

Plasmids. The luciferase promoter construct driven by −908 bp of human adiponectin promoter [p(-908)/LUC wt] and mutated construct [p(-908)/LUC PPRE mut] containing mutations in PPAR response element (PPRE) were prepared as described previously [21]. Other plasmids were purchased from Promega (Madison, WI).

Cell culture. 3T3-L1 pre-adipocytes were grown to confluence and induced to differentiate into mature adipocytes, as described previously [21].

Quantitative real-time PCR. Total RNA from 3T3-L1 cells and tissues were extracted using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan). The cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR was performed on the ABI7900 using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), according to the protocol supplied by the manufacturer. The sequences of primers used in real-time PCR are listed in Supplementary Table 1.

Promoter analysis of human adiponectin gene. On day 4 after induction of differentiation, 3T3-L1 cells in 6-well plates were transfected with 1 μg of the luciferase reporter plasmids along with 0.5 μg of pCMX-β-gal (internal standard) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the protocol recommended by the manufacturer. Four hours later, the cells were incubated with DMEM containing NO donor for 20 h, and luciferase activities were assayed using Luciferase Assay System (Promega, Madison, WI). Luciferase values were normalized by an internal β-galactosidase control and expressed as the relative luciferase activity.

Animals. C57BL/6J mice were purchased from CLEA Japan. For diet-induced obesity studies, female C57BL/6J mice were divided at random into two groups at 8 weeks of age. The first group was fed high-fat diet containing 30% fat by weight based on AIN93G while the second group was fed normal chow containing 5.9% fat by weight (CRF-1; Oriental Yeast Co.) for 20 weeks. Mice of the two groups were sacrificed at 7 months of age. Parametrial WAT was dissected out and frozen until analysis.

Measurement of nitrate/nitrite levels in plasma. Nitrite levels in plasma were measured using Nitric Oxide Assay Kit (Calbiochem).

Protein modifications and biotin switch technique. Biotin switch assays were performed as described previously [22]. Samples were subjected to SDS–PAGE, and biotinylated proteins were detected using horseradish peroxidase (HRP)-conjugated streptavidin (BD Biosciences, Bedford, MA). Equal protein loading and transfer were reconfirmed with Ponseau staining (Sigma).

Statistical analysis. All data were expressed as means ± SEM. Differences between groups were examined for statistical significance using the Student's *t*-test. A P value less than 0.05 denoted the presence of statistically significant difference.

Results

Effects of NO on gene expression levels in 3T3-L1 adipocytes

To examine the effects of NO on gene expression levels, 3T3-L1 adipocytes were incubated with NOR5, a spontaneous NO releaser with a half-life time of 20 h. The concentration of nitrite in the culture medium treated with vehicle and 0.1 mM NOR5 for 20 h was 3.0 ± 0.91 and 58.0 ± 11.4 μM, respectively. To eliminate the potential effect of cytotoxicity of NOR5, we analyzed LDH release in the culture media of 3T3-L1 adipocytes, and the results indicated that 0.05–1 mM of NOR5 did not increase LDH release (data not shown). Quantitative real-time PCR demonstrated that NOR5 treatment significantly reduced the mRNA expression levels of adiponectin, PPARγ, adipose P2 (aP2), lipoprotein lipase (LPL), and leptin, whereas it induced those of peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1), PAI-1 and IL-6, compared to vehicle treatment, in a dose-dependent manner (Fig. 1). Similar effects were observed with *S*-nitrosoglutathione, another NO donor (data not shown).

Effects of NO on secretion and promoter activity of adiponectin in 3T3-L1 adipocytes

NOR5 treatment reduced adiponectin protein released in the medium of 3T3-L1 adipocytes (Fig. 2A). To clarify the upstream factor responsible for this reduction, protein levels of PPARγ, a critical transcription factor for adiponectin [21], were measured. Expression levels of PPARγ protein decreased significantly by NOR5 treatment (Fig. 2B). Next, to assess the effect of NO on the transcription of adiponectin, we measured luciferase activity driven by human adiponectin promoter in 3T3-L1 adipocytes with or without NOR5. NOR5 treatment reduced basal luciferase activity of wild-type adiponectin promoter [p(-908)/LUC wt] by 6-fold ($P < 0.01$), but of SV40 promoter (pGL3-promoter) by only 1.1-fold (Fig. 2C). To examine the role of PPARγ on reduction of luciferase activity of adiponectin promoter by NOR5 treatment, luciferase activity driven by human adiponectin promoter with PPRE mutant [p(-908)/LUC PPRE mut] was measured in 3T3-L1 adipocytes treated with or without NOR5. The mutant promoter showed lower luciferase activity than wild-type promoter, however, the promoter activity did not change significantly with NOR5 treatment (Fig. 2C).

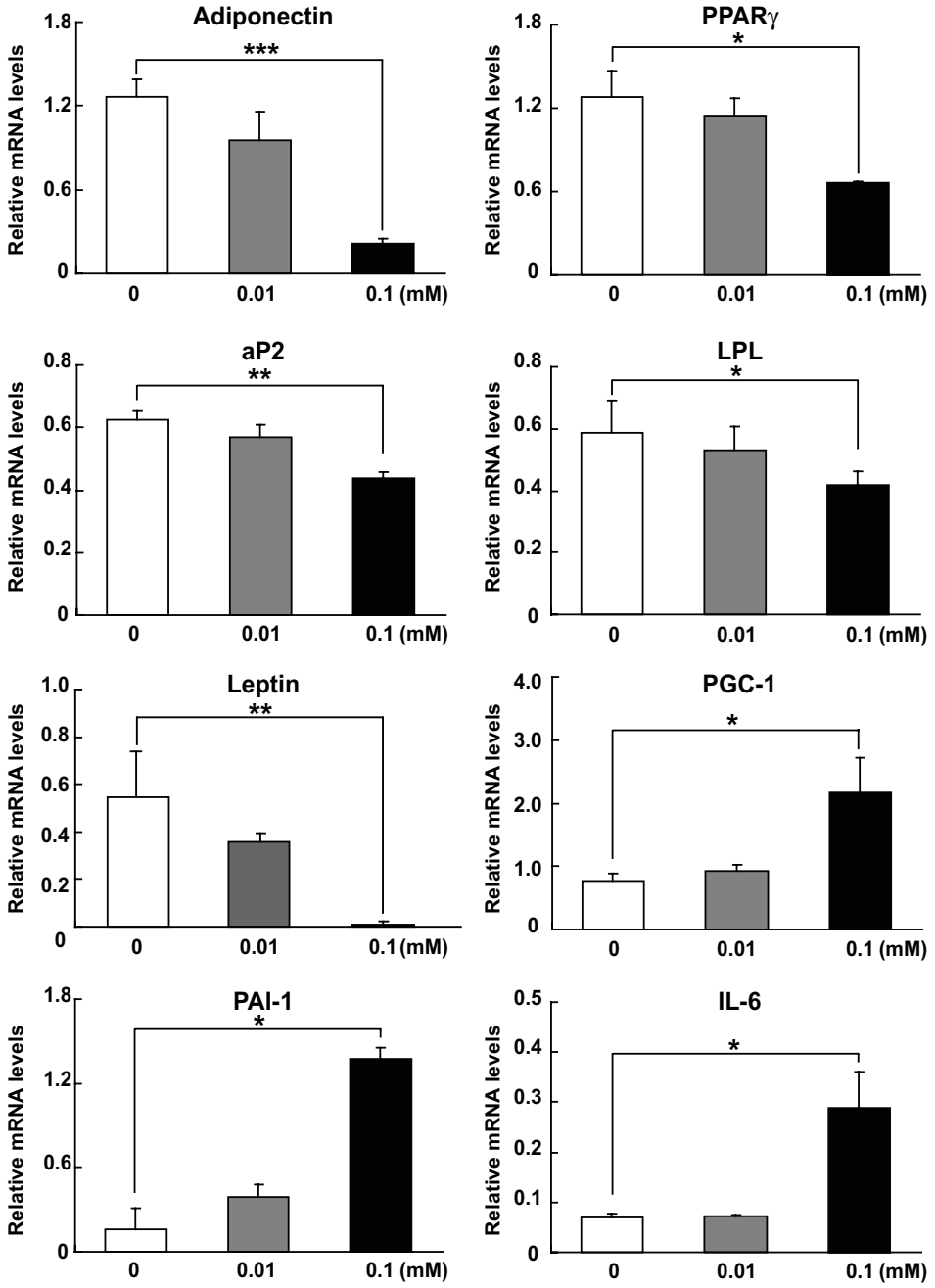

Fig. 1. Effects of NO on 3T3-L1 adipocytes. After their differentiation for 7 days, 3T3-L1 adipocytes were incubated for 20 h with the indicated concentration of NOR5. The mRNA expression levels of adiponectin, PPARγ, aP2, LPL, leptin, PGC-1, PAI-1, and IL-6 were quantified by real-time PCR. Values are normalized to the level of cyclophilin mRNA and expressed as means ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Signaling pathways of NO, and effects of inhibitors on NO-induced dysregulation of adipocytokines

To examine whether NO activates JNK, MAPK, and nuclear factor-kappa B (NF-κB) pathways, 3T3-L1 adipocytes were incubated with NOR5. Western blot analysis demonstrated that NOR5 treatment induced phosphorylation of JNK and degradation of IκB in 3T3-L1 adipocytes, but no change in p38 MAPK phosphorylation (Supplementary Fig. 1). Next, to investigate the signaling pathway responsible for NO-mediated dysregulation of gene expressions, 3T3-L1 adipocytes were treated with SP600125, a JNK inhibitor or BAY11-7085, an IKK inhibitor. Both inhibitors reversed NOR5-mediated induction of PAI-1 and IL-6 mRNA expressions, but did not restore adiponectin and PPARγ mRNA expressions (Fig. 3A and B). To examine the significance of guanylate cyclase pathway, 3T3-L1 adipocytes were treated with ODQ, a guanylate cyclase inhibitor. As reported previously, ODQ blocked NOR5-mediated upregulation of PGC-1 expression [23] (Supplementary Fig. 2A), however, ODQ had no effect on NOR5-mediated changes in adiponectin, PPARγ,

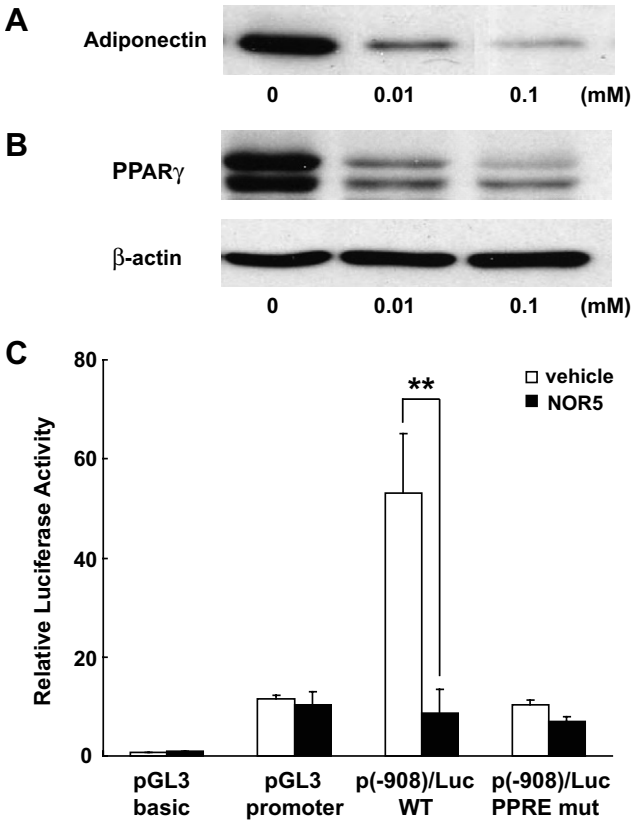

Fig. 2. NO downregulates adiponectin and PPARγ expressions. 3T3-L1 adipocytes were incubated for 20 h with the indicated concentration of NOR5. (A) Conditioned medium (4 μl/lane) and (B) cell lysates (30 μg/lane) were analyzed by Western blot using the indicated antibodies. (C) 3T3-L1 cells were transfected with luciferase constructs lacking promoter (pGL3-basic), having SV40 promoter (pGL3-promoter), adiponectin promoter [p(-908)/LUC wt] or mutated promoter [p(-908)/LUC PPRE mut]. After 4 h, the transfected cells were incubated for 20 h with vehicle or 0.05 mM NOR5. Luciferase activities were measured and normalized by β-galactosidase activity. Data are means ± SEM ($n = 3$). $^{**}P < 0.01$.

PAI-1 and IL-6 mRNA expressions (Supplementary Fig. 2A, and data not shown). To assess the contribution of ROS on NOR5-mediated change in adipocytokine mRNA expressions, 3T3-L1 adipocytes were treated with NAC, a general antioxidant. As reported previously, NAC blocked ROS donor (HX/XO)-mediated downregulation of adiponectin expression [24] (Supplementary Fig. 2B), but had no effect on NOR5-mediated change in adiponectin, PPARγ, PAI-1, and IL-6 mRNA expressions (Supplementary Fig. 2B, and data not shown).

Induction of NO synthesis in WAT of high-fat diet-induced obese mice

To examine NO production in obese mice, we compared C57BL/6J mice fed normal chow (control) and those fed high-fat diet. Plasma levels of nitrate/nitrite, stable metabolites of NO, were higher in high-fat diet mice than in control mice (Fig. 4A). The levels of *S*-nitrosylated proteins were higher in WAT of high-fat diet mice than the control mice (Fig. 4B). Analysis of mRNA expression showed that eNOS and iNOS mRNA levels were significantly elevated in WAT of obese mice than in control mice, in parallel with increased *S*-nitrosylated proteins. In high-fat diet mice, the mRNA levels of adiponectin, and PPARγ were decreased, whereas those of PAI-1, IL-6, and monocyte chemoattractant protein-1 (MCP-1) were increased, relative to the control mice (Fig. 4C). The mRNA levels of macrophage infiltration marker gene, CD68, were significantly upregulated in WAT of high-fat-fed HF mice than control mice (Fig. 4C).

Discussion

Recent investigations have revealed the involvement of NO in the development of insulin resistance in obesity models [17,18], however, it has not been fully determined whether NO might have direct effects on adipocytokine expressions. We demonstrated for the first time that NO reduced gene expression and secretion of adiponectin. Promoter analysis indicated that reduction of adiponectin expression might be dependent on downregulation of PPARγ. To investigate the mechanism to downregulate PPARγ, 3T3-L1 adipocytes were treated with inhibitors of JNK, NF-κB, and guanylate cyclase. However, none of these inhibitors could reverse the NO-mediated downregulation of PPARγ.

Another mechanism of NO to regulate protein expression and function is NO-dependent modifications. NO is known to react with superoxide and other ROS to produce peroxynitrite, a highly cytotoxic reactive nitrogen species, which induces cysteine *S*-nitrosylation and tyrosine nitration of proteins [25]. Shibuya et al. reported that peroxynitrite treatment induced nitration of PPARγ. This treatment inhibited translocation of PPARγ into the nucleus, but did not alter its protein amount in RAW264 macrophages [26]. Our results demonstrated that NOR5 treatment reduced PPARγ expressions at mRNA level (Fig. 1A), and treatment with NOR5 and NAC, a scavenger of oxidative stress, did not restore mRNA levels of PPARγ (Fig. 4B). These results suggest that, in addition to protein modification, PPARγ expression should be regulated at mRNA level. To reveal this, we measured DEC1 expressions, a known factor to reduce PPARγ mRNA expressions [27], however, they showed no change in 3T3-L1 adipocytes treated with or without NOR5 (data not shown). Further study is needed to elucidate the molecular mechanism of NO-mediated downregulation of PPARγ.

In other types of cells, it is reported that NO regulates phosphorylation of JNK and p38 MAPK [28–30], and activates NF-κB signaling pathways [31–33]. We demonstrated for the first time that NO activated JNK, and NF-κB pathways, but not p38 MAPK in 3T3-L1 adipocytes (Fig. 2A). In the current study, NO-induced mRNA levels of PAI-1 and IL-6, and these inductions were reversed by inhibitors of JNK or NF-κB pathways. These results indicated that,

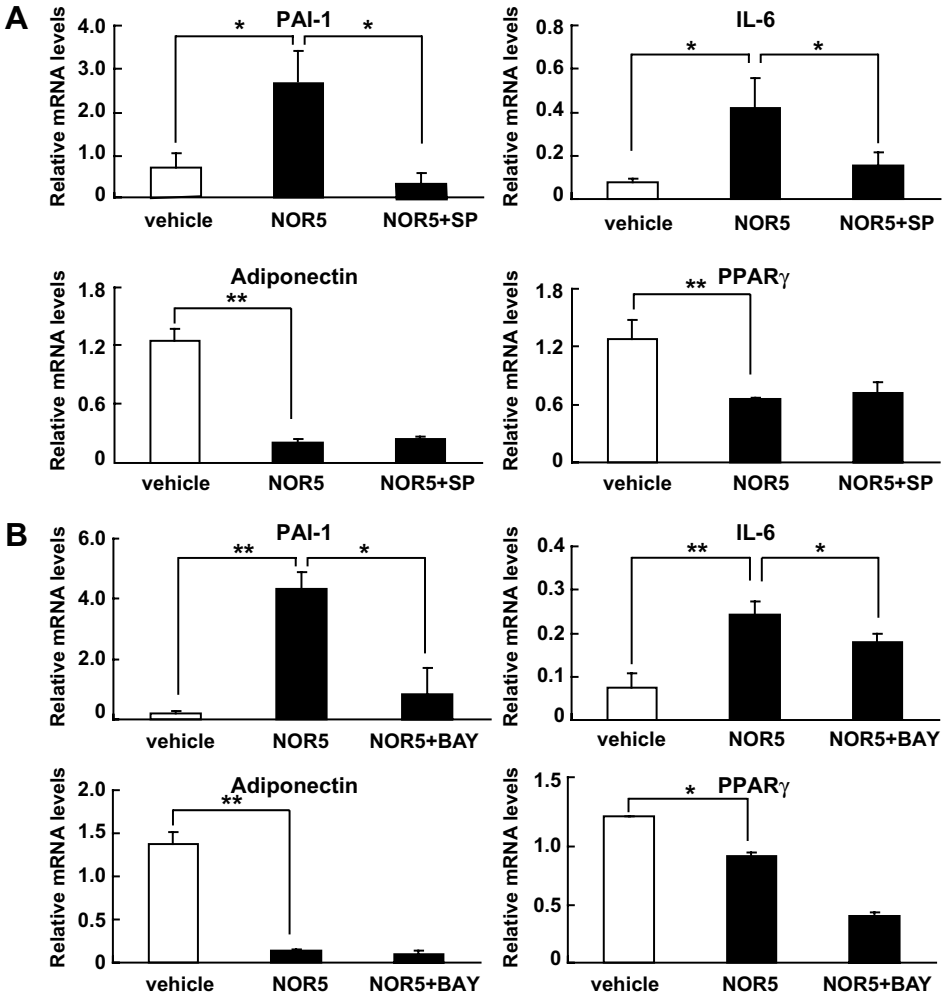

Fig. 3. Effects of JNK and NF-κB inhibitors on the NO-mediated dysregulation of gene expressions. (A,B) 3T3-L1 adipocytes were incubated with or without (A) 20 μM SP600125 (SP), (B) 10 μM BAY11-7085 (BAY) for 30 min, followed by treatment with vehicle or 0.1 mM NOR5 for 20 h. The mRNA expression levels of PAI-1, IL-6, adiponectin, and PPARγ were quantified by real-time PCR. Values are normalized to the level of cyclophilin mRNA and expressed as means ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$.

in 3T3-L1 adipocytes, NO-induced IL-6 and PAI-1 expressions through NF-κB- and JNK-dependent pathways.

Using the biotin switch assay, we revealed for the first time the presence of high levels of *S*-nitrosylated proteins in WAT of obese mice on high-fat diet. Considering the direct effect of NO on adipocytes *in vitro*, elevated NO synthesis in WAT may result in downregulation of adiponectin *in vivo*. However, previous *in vivo* studies found no difference in serum adiponectin levels between control and iNOS-deficient mice [34], and that NOS inhibitors had no effects on adiponectin mRNA levels in WAT or serum adiponectin levels [18,35]. The reason for the discrepancy between these reports *in vivo* and our results *in vitro* is unknown at present. In WAT of obese, other factors suppressing adiponectin expression are increased, such as hormones [36], free fatty acids [37], and ROS [24]. Impact of NO on adiponectin expression might be small, compared to these factors *in vivo*.

As for inflammatory cytokines, we demonstrated that NO-induced PAI-1 and IL-6 mRNA expressions in 3T3-L1 adipocytes, suggesting that inhibition of NOS activity may improve the inflammation in WAT. In this regard, our results *in vitro* are supported by the recent paper, showing reduced inflammation in WAT by treatment with NG-nitro-L-arginine methyl ester, a NOS inhibitor, in high-fat-fed obese mice *in vivo* [35]. NO might play an important role in WAT of obese mice through dysregulation of inflammatory cytokines.

Taken together, we demonstrate suppression of adiponectin, and induction of PAI-1 and IL-6 expressions by NO in 3T3-L1 adipocytes. We present evidence that JNK and NF-κB pathways mediate inductions of PAI-1 and IL-6. Furthermore, NO synthesis is elevated in WAT of obese mice with upregulation of PAI-1 and IL6, and downregulation of adiponectin. Our results suggest that increased NO synthesis might be partly responsible for dysregulation of adipocytokines in WAT.

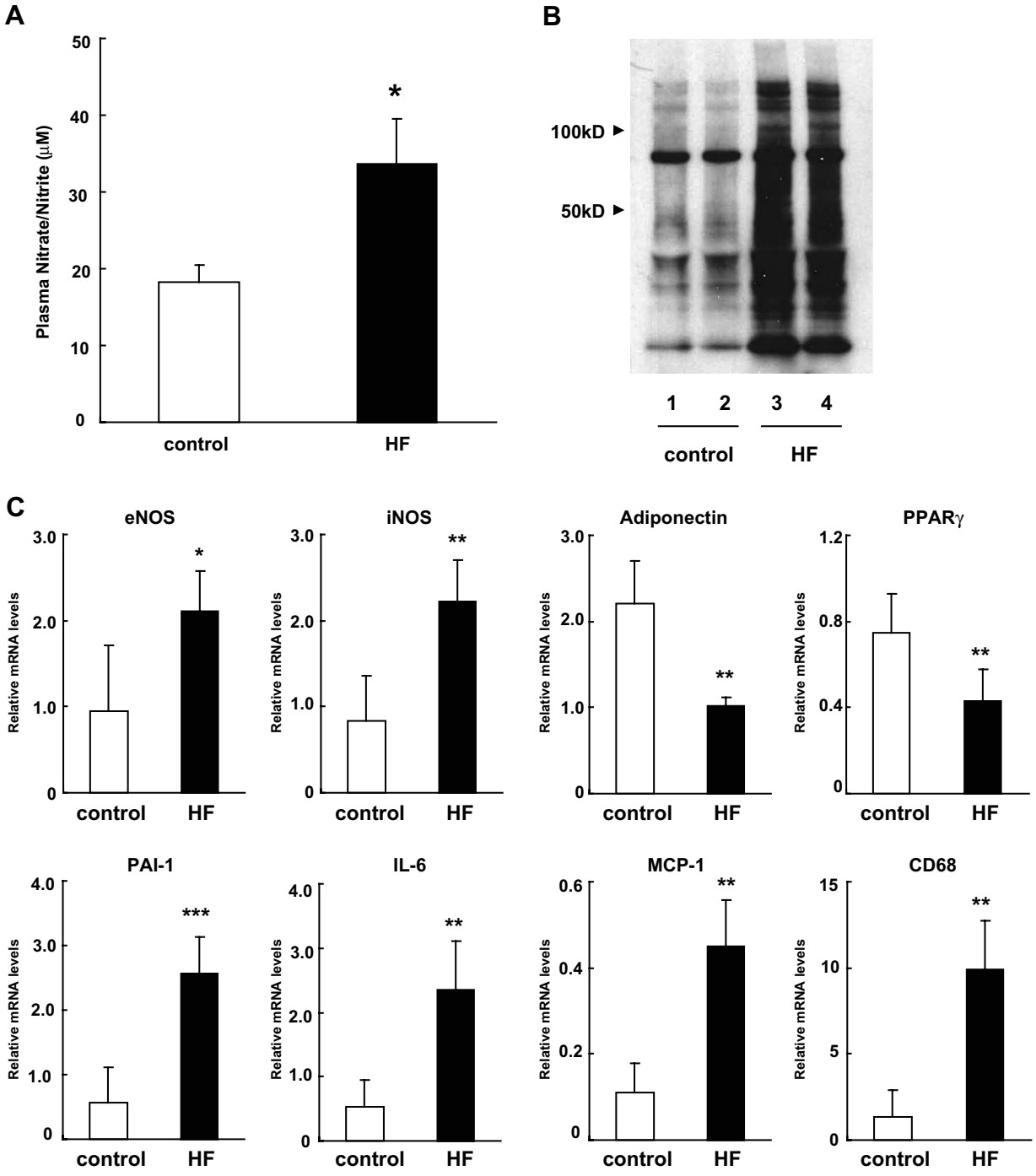

Fig. 4. Measurement of NO and gene expressions in diet-induced obese mice. (A) Plasma levels of nitrate/nitrite in mice fed normal diet (control, $n = 6$) and high-fat diet (HF, $n = 6$). Values are expressed as means ± SEM. $^{*}P < 0.05$ compared with control group. (B) Amount of *S*-nitrosylated proteins in WAT. WAT from two normal diet fed mice (control; lanes 1 and 2) and two high-fat diet-fed mice (HF; lanes 3 and 4) were subjected to the biotin switch assay. Equal protein loading and transfer were reconfirmed with Ponseau staining. (C) The mRNA levels of eNOS, iNOS, adiponectin, PPARγ, PAI-1, IL-6, MCP-1, and CD68 in WAT of normal diet-fed (control) and high-fat diet-fed (HF) mice were quantified by real-time PCR. Values are normalized to the levels of cyclophilin mRNA and expressed as means ± SEM ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.09.084.

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