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Reciprocal regulation of natriuretic peptide receptors by insulin in adipose cells

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ABSTRACT

Atrial- and brain-type natriuretic peptides (ANP and BNP, respectively) have been shown to exert potent lipolytic action in adipocytes. A family of natriuretic peptide receptors (NPRs), NPR-1, NPR-2, and NPR-3, mediates their physiologic effects. NPR-1 and NPR-2 are receptor guanylyl cyclases, while NPR-3 lacks enzymatic activity and functions primarily as a clearance receptor for natriuretic peptides. ANP has a high affinity for NPR-1 and NPR-3 than other natriuretic peptides. There is a possibility that ANP may exhibit its lipolytic effect through the balance of NPR-1 and NPR-3 expressions in adipocytes. However, the regulation of adipose NPRs has not been fully elucidated. We here examined the regulation of mouse adipose NPRs by insulin, an anti-lipolytic hormone. Among the insulin target organs, NPR-1 mRNA levels were higher in white adipose tissue (WAT) than in liver and skeletal muscle. NPR-3 mRNA was expressed most abundantly in WAT. Fasting condition induced NPR-1 mRNA level while suppressed NPR-3 mRNA level in WAT. Administration of streptozotocin resulted in the increase of NPR-1 mRNA level while the decrease of NPR-3 mRNA level in WAT. In *ob/ob* mice, hyperinsulinemic model, NPR-1 mRNA level was lower whereas NPR-3 mRNA level was higher compared to lean control mice. In 3T3-L1 adipocytes, insulin significantly reduced NPR-1 mRNA level while increased NPR-3 mRNA levels both through phosphatidylinositol 3-kinase (PI3-kinase) pathway. In summary, NPR-1 and NPR-3 were highly expressed in WAT and adipose NPR-1 and NPR-3 were reciprocally regulated by insulin. This study suggests that insulin may efficiently promote lipogenesis partly by reducing the lipolytic action of ANP through the opposite regulation of NPR-1 and NPR-3.

Introduction

Adipocytes release free fatty acid (FFA) and glycerol into the blood stream by hydrolyzing intracellular triglycerides (TGs) under starvation and/or sympathetic nerve-activation state [1–3]. During lipolysis, intracellular TGs undergo hydrolysis through the sequential action of lipases such as adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase (MGL). The metabolic switch between lipolysis versus lipogenesis is mainly regulated by several hormones including insulin and catecholamines. In adipocytes, insulin has been shown to possess a potent lipogenic effect. In the fed state, insulin inhibits lipolysis through the phosphatidylinositol 3-kinase (PI3-kinase)-Akt-phosphodiesterase-3B (PDE3B) pathway with a consequent decrease in intracellular cyclic AMP (cAMP) and promotes glucose uptake into adipocytes through glucose transporter 4 (GLUT4). Conversely, in fasting condition, catecholamines promote lipolysis via the activation of adrenoreceptors by increasing intracellular cAMP levels through adenyl cyclase.

Atrial- and brain-type natriuretic peptides (ANP and BNP, respectively) have been shown to exert potent lipolytic action by increasing intracellular cyclic GMP (cGMP) in adipocytes [4]. A family of natriuretic peptide receptors (NPRs), NPR-1, NPR-2, and NPR-3, mediates their physiologic effects. ANP and BNP bind NPR-1 while C-type natriuretic peptide (CNP) signals through NPR-2 [5]. NPR-1 and NPR-2 are receptor guanylyl cyclases known as NPR-A/GC-A and NPR-B/GC-B, respectively, while NPR-3 (NPR-C) lacks enzymatic activity and functions primarily as a clearance receptor. ANP has a high affinity for NPR-1 and NPR-3 than other natriuretic peptides while CNP preferentially binds NRP-2 [6].

Taken together, ANP may exhibit its lipolytic action through the local balance of NPR-1 and NPR-3 expressions in adipocytes. However, the regulation of NPRs has not been fully understood in adipocytes. We here examined the regulation of mouse adipose NPRs by focusing on insulin, an anti-lipolytic hormone.

Materials and methods

Animal preparation. Male B6.V-*Lep*^{ob}/J (*ob/ob*) and their respective lean control male C57BL/6J mice were purchased from Charles River Laboratories (Charles River Japan, Inc., Yokohama, Japan). For the experiment on fasting and ad libitum feeding, C57BL/6J mice (n = 6

Abbreviations: NPR, natriuretic peptide receptor; NEP, neutral endopeptidase; WAT, white adipose tissue; PI3-kinase, phosphatidylinositol 3-kinase.

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per group) were used at 7 weeks of age. The fasting group (Fast) was deprived of chow for 24 h before sacrifice. The ad libitum feeding group (Adlib) was allowed free access to standard laboratory chow. For the experiment of insulin deficiency, C57BL/6J mice were intraperitoneally administered with 150 mg/kg of streptozotocin (STZ, Sigma Chemicals Co., St. Louis, MO, USA) dissolved in citrate buffer (0.05 mol/L citric buffer, pH 4.5) at 6 weeks of age under overnight fasting condition. On day 7 after the STZ injection, mice were killed and analyzed. Plasma glucose and insulin were measured by the Glucose CII-Test (Wako Pure Chemical, Osaka, Japan) and Ultra Sensitive Insulin ELISA Kit (Morinaga, Kanagawa, Japan), respectively. Mice were acclimated to the new environment for a week before the experiments and were kept in rooms set at 22 °C with a 12–12 h dark–light cycle (light cycle, 8 AM to 8 PM). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

3T3-L1 cell cultures. 3T3-L1 cells were maintained and differentiated as described previously [7]. Briefly, cells were grown to confluence and differentiated by induction media (Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) containing 0.5 mM of 1-methyl-3-isobutylxanthine, 1 μM of dexamethasone, and 5 μg/mL of insulin) for 48 h. After incubation with the induction media for 48 h, media was changed to maintenance media (DMEM supplemented with 10% FCS). On day 7 after differentiation, media was changed to DMEM containing 0.5% fatty acid free bovine serum albumin (BSA) for 12 h and then 3T3-L1 adipocytes were sequentially treated with 10 μM of LY294002 (Calbiochem, San Diego, CA, USA) or 10 μM of PD98059 (Wako Pure Chemical, Osaka, Japan) or without compounds. After treatment with these compounds for 1 h, cells were incubated with insulin at the indicated dosage and time and, were harvested. LY294002 and PD98059 were used as an inhibitor of phosphatidylinositol 3 kinase (PI3-kinase) and an inhibitor of mitogen-activated protein kinase kinase (MEK), respectively.

Quantification of mRNA levels. Total RNA was isolated from mouse tissues by using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) according to the protocol supplied by the manufacturer. The quality and quantity of total RNA were determined by using ND-1000 Spectrophotometer (Nano Drop Technologies, Wilmington, DE). First-strand cDNA was synthesized from 240 ng of total RNA using Thermoscript RT (Invitrogen) and oligodT primer. Real-time quantitative PCR amplification was conducted with the LightCycler 1.5 (Roche Diagnostics, Mannheim, Germany) using LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics) according to the protocol recommended by the manufacturer. The results for each sample were normalized to the respective 36B4 mRNA levels. The following primers were used: NPR-1, 5′-GGCACTTGCACTGCTCGAT-3′ (forward) and 5′-TGACTGTGTCTCCAAAGAGGCA-3′ (reverse), NPR-2; 5′-TGACCAGCTGAGGTTACGCATA-3′ (forward) and 5′-GACATGGATCTTTAGAGCTTGGC-3′ (reverse), NPR-3; 5′-TCCATGGAGGTGAAAAGTTCTGTT-3′ (forward) and 5′-CCCCATCCTTCTTGCTGTAGC-3′ (reverse), neutral endopeptidase (NEP); 5′-CTCAGCCGAAACTACAAGGAGTC-3′ (forward) and 5′-CAGCAAAAGCCGCTTCCA-3′ (reverse), 36B4; 5′-GGCCAATAAGGTGCCAGCT-3′ (forward) and 5′-TGATCAGCCCGAAGGAGAAG-3′ (reverse).

Statistical analysis. Results were expressed as means ± SEM of *n* separate experiments. Differences between groups were examined for statistical significance using Student's *t*-test with Fisher's protected least significant difference test. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

Results

Tissue distribution of NPRs and NEP mRNA in mice

First, the tissue distribution of NPRs and neutral endopeptidase (NEP), a degradative enzyme of natriuretic peptides [8], were investigated. Abundant expression of NPR-1 mRNA level was observed in lung and testis, while NPR-1 mRNA level was also detected in white adipose tissue (WAT) (Fig. 1A). The levels of NPR-2 mRNA were detected in various tissues, especially in lung, brain, heart, eye, and WAT (Fig. 1B). Interestingly, predominant expression of NPR-3 mRNA was observed in WAT and its mRNA was also detected in lung (Fig. 1C). NEP mRNA levels were abundantly expressed in small intestine, kidney, and lung, but its mRNA level of WAT was lower than of these tissues (Fig. 1D).

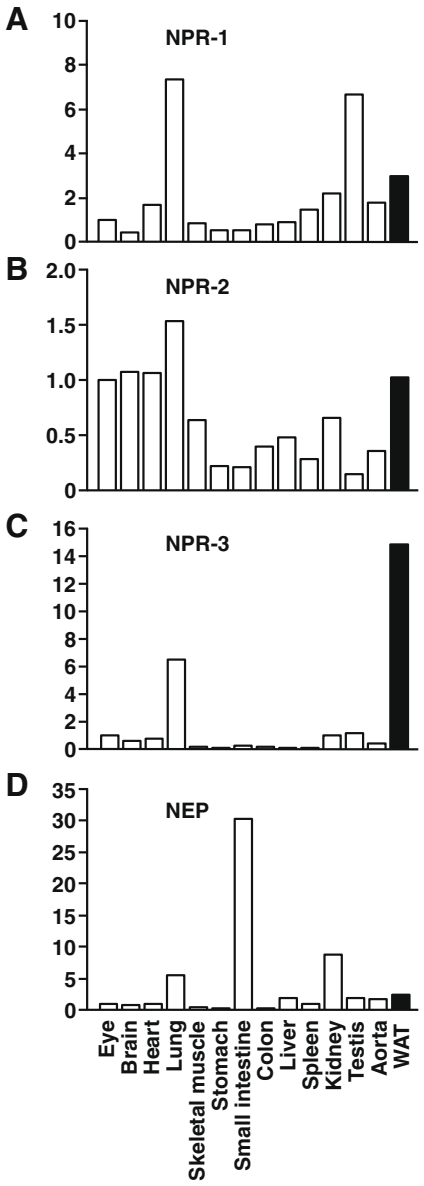

Fig. 1. Tissue distribution of NPRs and NEP in mice. Each organ was excised from C57BL/6J mice at 11 weeks of age. RT-PCR was performed as described in Materials and methods. Levels of mRNA expression are normalized in mRNA level of eye. (A) NPR-1, (B) NPR-2, (C) NPR-3, (D) NEP. WAT, white adipose tissue.

Regulation of NPRs in WAT

As shown in Fig. 1, mRNA levels of NPRs were substantially detected in WAT, but NEP mRNA level was low in WAT. We thus examined the mRNA changes of NPRs in WAT under several nutritional conditions. Fig. 2A–C shows mRNA levels of NPRs under ad libitum or fasting condition. Plasma levels of insulin under fasting condition were significantly decreased compared to ad libitum

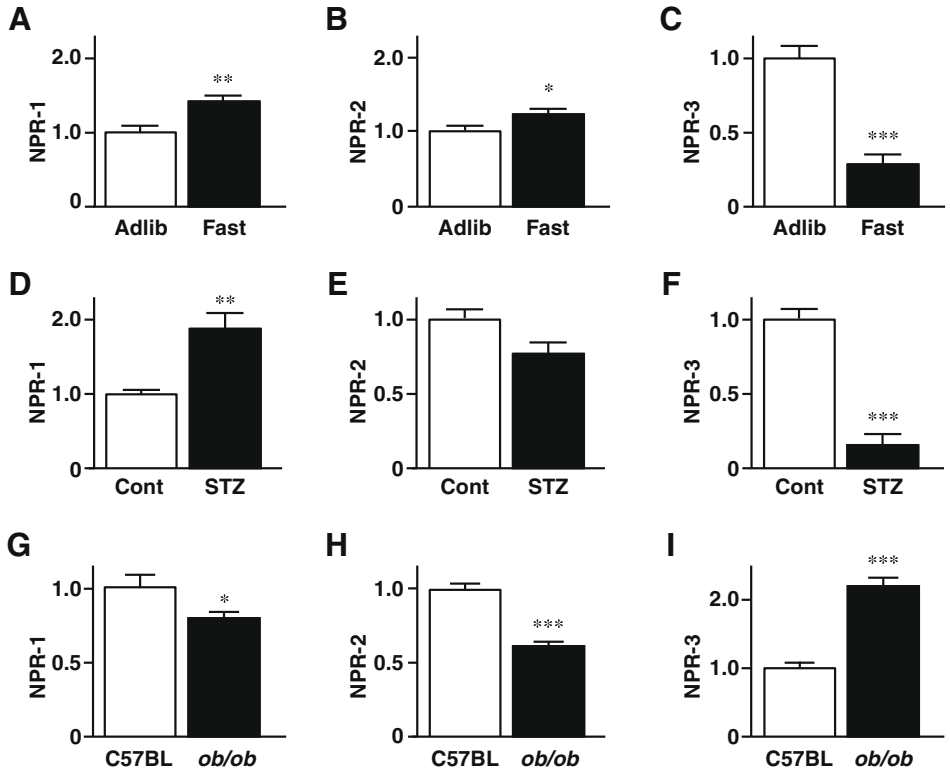

Fig. 2. Regulation of NPRs in white adipose tissue of mice. RT-PCR was performed and relative mRNA levels were calculated as described in Materials and methods. (A–C) Effect of fasting (Fast) and ad libitum (Adlib) conditions on NPRs mRNA levels. C57BL/6J mice ($n = 4$ per group) were analyzed at 7 weeks of age. (D–F) Adipose NPRs mRNA levels in STZ-treated (STZ) and STZ-untreated (Cont) mice ($n = 6$ per group). C57BL/6J mice were administered with 150 mg/kg of STZ and analyzed on day 7 after STZ injection. (G–I) Adipose NPRs mRNA levels in obese *ob/ob* mice and C57BL/6J mice (C57BL), as lean control mice ($n = 4$ per group). Mice were analyzed at 7 weeks of age. The data are expressed as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Adlib, Cont, and C57BL groups, respectively.

condition (0.175 ± 0.118 ng/mL in fasting condition and 0.975 ± 0.197 ng/mL in ad libitum condition, $P < 0.05$, $n = 4$). Plasma glucose concentrations were also significantly lower in fasting condition than in ad libitum condition (data not shown). NPR-1 and NPR-2 mRNA levels were significantly higher in fasted condition than in ad libitum (Fig. 2A and B), whereas NPR-3 mRNA level was markedly suppressed by fasting (Fig. 2C).

We next analyzed STZ-treated mice as an insulin deficient model. Administration of STZ significantly depleted plasma insulin levels (0.004 ± 0.004 ng/mL in STZ-treated mice and 0.737 ± 0.071 ng/mL in control mice (STZ-untreated mice), $P < 0.0001$, $n = 6$). Plasma glucose levels of STZ-treated mice were apparently higher than those of control mice (749.4 ± 61.0 mg/dL in STZ-treated mice and 160.3 ± 2.9 mg/dL in control mice, $P < 0.0001$, $n = 6$). NPR-1 mRNA level of STZ-treated mice was significantly higher than that of control mice (Fig. 2D), while NPR-3 mRNA level was dramatically lower in STZ-treated mice than in control mice (Fig. 2F). No significant changes of NPR-2 mRNA level were observed in these mice (Fig. 2E).

Next, male *ob/ob* mice were analyzed as a hyperinsulinemic obese model at 7 weeks of age. Male C57BL/6J mice were used as lean control mice at the same age. Plasma insulin levels of *ob/ob* mice were significantly higher than those of C57BL/6J mice (32.200 ± 4.863 ng/mL in *ob/ob* mice and 0.975 ± 0.197 ng/mL in C57BL/6J mice, $P < 0.001$, $n = 4$). Plasma glucose concentrations were also significantly higher in *ob/ob* mice than in C57BL/6J mice (275.4 ± 38.3 mg/dL in *ob/ob* mice and 135.3 ± 4.4 mg/dL in C57BL/6J mice, $P < 0.05$, $n = 4$). NPR-1 and NPR-2 mRNA levels of *ob/ob* mice were lower than those of C57BL/6J mice (Fig. 2G and H), while NPR-3 mRNA level was significantly higher in *ob/ob* mice compared to C57BL/6J mice (Fig. 2I).

Regulation of NPRs by insulin in 3T3-L1 adipocytes

As demonstrated in Fig. 2, insulin was suggested to regulate oppositely the adipose mRNA levels of NPR-1 and NPR-3. We thus investigated the direct effects of insulin on adipose NPR-1 and NPR-3 mRNA levels by using 3T3-L1 differentiated-adipocytes. No apparent changes of NPR-1 mRNA level were observed through 3T3-L1 adipocytes differentiation (Fig. 3A), while NPR-3 mRNA level was increased during the 3T3-L1 adipocytes differentiation (Fig. 3B). Insulin significantly suppressed NPR-1 mRNA levels in various concentrations (Fig. 3C), while NPR-3 mRNA levels were significantly elevated by insulin in a dose-dependent manner (Fig. 3D). The time-dependent changes of NPR-1 and NPR-3 mRNA levels were also examined by using 10^{-9} M of insulin. Insulin significantly decreased NPR-1 mRNA levels after 3 h and its decrease was maintained for 12 h (Fig. 3E). Administration of insulin rapidly elevated NPR-3 mRNA levels and its increase was observed for 12 h (Fig. 3F).

It is well known that insulin enhances two signal pathways: Ras/extracellular signal-regulated kinase 1/2 (ERK1/2) and PI3-kinase/Akt pathway. To clarify the pathway in which the insulin signal is involved in the effect on NPR-1 and NPR-3 mRNA regulations, PD98059 (MEK inhibitor) and LY294002 (PI3-kinase inhibitor) were used. Insulin treatment decreased NPR-1 mRNA levels (Fig. 4A, lane 1 versus 3), but its decrease was not recovered by PD98059 (Fig. 4A, lane 3 versus 4). Treatment with LY294002 significantly abolished the inhibitory effect of insulin on NPR-1 mRNA levels (Fig. 4B, lane 3 versus 4). Similarly, NPR-3 mRNA level was increased by insulin (Fig. 4C, lane 1 versus 3), while the treatment with PD98059 did not affect on insulin-induced elevation of NPR-3 mRNA levels (Fig. 4C, lane 3 versus 4). On the other hand, the insulin-dependent elevation of NPR-3 mRNA levels was significantly attenuated by the

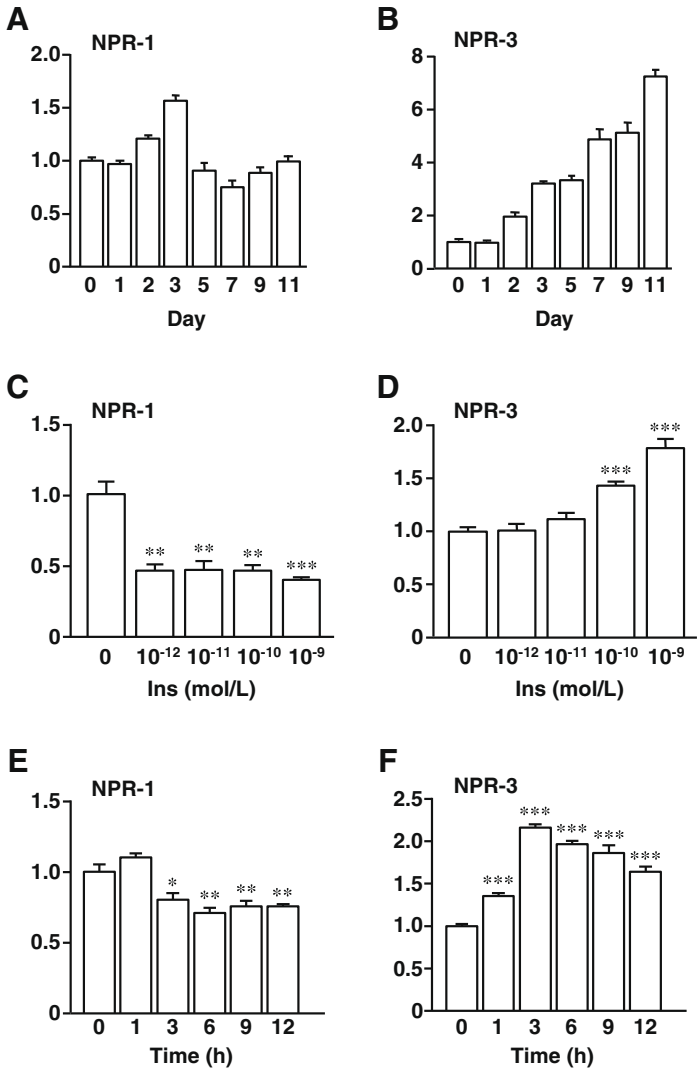

Fig. 3. Effect of insulin on NPR-1 and -3 in 3T3-L1 adipocytes. 3T3-L1 adipocytes were maintained, RT-PCR was performed, and the relative mRNA levels were calculated as described in Materials and methods. (A,B) Changes of mRNA levels of NPR-1 and -3 under 3T3-L1 adipocytes differentiation. (C,D) Dose-dependent effects of insulin (Ins) on NPR-1 and -3 mRNA levels. On day 7 after differentiation, 3T3-L1 adipocytes were starved and treated with the indicated dosage of insulin for 3 h. (E,F) Time-dependent effects of insulin on NPR-1 and -3 mRNA levels. On day 7 after differentiation, 3T3-L1 adipocytes were starved and treated with 10^{-9} mol/L of insulin. The data are expressed as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus 0 mol/L of insulin (C,D) or 0 h (E,F).

treatment with LY294002 (Fig. 4D, lane 3 versus 4). These results suggest that insulin suppresses NPR-1 mRNA level while elevates NPR-3 mRNA level both through PI3-kinase pathway.

Discussion

As shown in Fig. 1A and C, it is worthy of remark that expression levels of NPR-1 and NPR-3 in WAT were higher than in other insulin-sensitizing organs, liver and skeletal muscle. We demonstrated for the first time that insulin oppositely regulates NPR-1 and NPR-3 in adipocytes. Insulin, an anti-lipolytic hormone, reduced NPR-1 mRNA level while elevated NPR-3 mRNA level, resulting that the lipolytic action of ANP may be attenuated. Together, insulin may efficiently promote lipogenesis in adipocytes partly by reducing the lipolytic effect of ANP through the insulin-mediated reciprocal regulations of NPR-1 and NPR-3.

The regulations of NPR-3 have been reported in several cell lines except the fat cell. Transforming growth factor-β1 (TGF-β1) elevates NPR-3 mRNA at the transcriptional level in murine thymic stromal cell line [9]. Fibroblast growth factors (FGF-1 and FGF-2) and platelet-derived growth factor BB (PDGF-BB) were shown to reduce the expression of NPR-3 mRNA in pulmonary arterial smooth muscle cells through MEK-ERK pathway [10]. In cultured mesangial cells, epidermal growth factor (EGF) have been reported to decrease NPR-3 mRNA, while PDGF and insulin-like growth factor-1 (IGF-1) were ineffective in reducing the levels of NPR-3 mRNA [11]. Several cytokines thus regulate NPR-3 in other cells while the regulation of NPR-3 has not been elucidated in adipocytes. Considering that NPR-3 is abundantly expressed in adipocytes and adipose tissues, further analyses for NPR-3 regulation in adipocytes may be meaningful in future.

Several investigators have reported the relation of obesity and plasma natriuretic peptides, but the results were inconstant [12–15]. Present study showed the elevation of NPR-3 mRNA level in obese WAT as demonstrated in Fig. 2I. Other group recently demonstrated similar results that NPR-3 mRNA level of WAT was increased when mice were fed with high-fat diet [16]. These results suggest that the lipolytic effect of natriuretic peptides may be locally attenuated in obese WAT, even though plasma

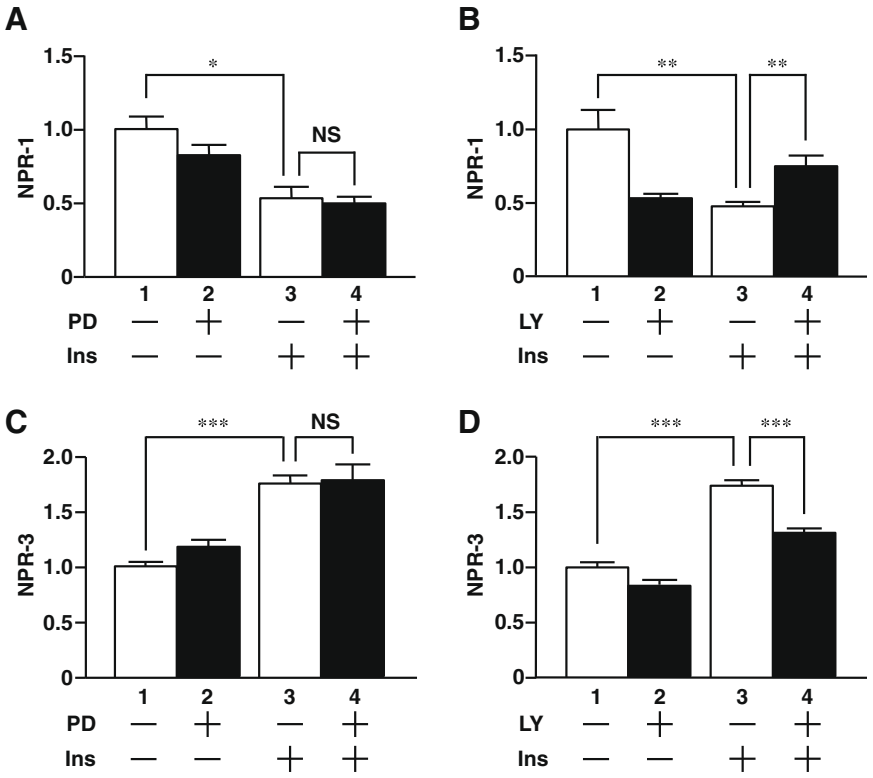

Fig. 4. Effects of inhibitors for insulin signaling on NPR-1 and -3 mRNA levels in 3T3-L1 adipocytes. 3T3-L1 adipocytes were maintained, RT-PCR was performed, and the relative mRNA levels were calculated as described in Materials and methods. On day 7 after differentiation, 3T3-L1 adipocytes were starved for 12 h and were sequentially treated with 10 μM of PD98059 (PD) (A,C) or 10 μM of LY294002 (LY) (B,D). After treatment with these compounds for 1 h, cells were incubated with 10^{-9} mol/L of insulin (Ins) for 6 h and were collected. (A,B) Changes of NPR-1 mRNA levels. (C,D) Changes of NPR-3 mRNA levels. The data are expressed as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NS indicates not significant.

levels of natriuretic peptides are not changed in obesity [12–15]. Our group recently showed that natriuretic peptides enhanced adiponectin production in human adipocytes through NPR-1/cGMP-dependent pathway [17], suggesting that the elevation of NPR-3 in obese WAT may cause a hypoadiponectinemia in a part. Further examination for adipose NPRs expression levels in obese human is needed in the future. NPR-3 antagonists may be one of useful therapeutic strategies for obesity-related disorders.

Conflict of interest

None declared.

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