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Anti-inflammatory and Insulin Signaling Phenotype Induced by Repeated Lipopolysaccharide Stimulation in 3T3-L1 Adipocytes

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Abstract. *Background/Aim: Lipopolysaccharide (LPS) is thought to be a causative agent of type 2 diabetes, because it has been shown that a single LPS stimulation in vitro induces chronic inflammation and reduces insulin signaling in adipocytes. However, oral LPS administration prevents type 2 diabetes, and this effect does not correspond to a single LPS adipocyte stimulation. In this study, the response of adipocytes to single and repeated stimulation with LPS was examined. Materials and Methods: 3T3-L1 cells were differentiated into adipocytes and stimulated with LPS once or thrice every 24 h. The expression levels of inflammatory and anti-inflammatory factors and insulin sensitivity-related factors were measured. Results: Single stimulation with LPS increased the mRNA and protein expression of inflammatory factors (interleukin-6 and monocyte chemotactic protein 1), but this increase was inhibited by repeated stimulation. In contrast, the mRNA expression levels of anti-inflammatory factors (proliferator-activated receptor γ and peroxisome proliferator-activated receptor gamma coactivator1 α) were increased by repeated LPS stimulation. Additionally, the mRNA expression levels of insulin sensitivity-related factors (glucose transporter type 4, insulin receptor, insulin receptor substrate 1 and thymoma viral proto-oncogene 2) in adipocytes were increased upon repeated LPS stimulation. Conclusion: Repetitive LPS stimulation, unlike single stimulation of adipocytes, upregulates anti-inflammatory and insulin signaling-related factors.*

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It has been hypothesized that lipopolysaccharide (LPS) transferred from the intestinal tract into the blood by a high-fat diet can contribute to the development of type 2 diabetes. In particular, LPS can induce the expression of inflammatory factors, including interleukin-6 (IL-6), and monocyte chemotactic protein 1 (Mcp-1), causing chronic inflammation, and also reduces expression of insulin signaling-related factors, including glucose transporter type 4 (Glut4) in adipocytes (1-3). In support, it has been shown that a single LPS stimulation *in vitro* induces inflammatory factors and decreases insulin signaling-related factors in adipocytes (4-6). However, it has also been found that oral LPS administration exerts anti-inflammatory effects, and additionally, has preventive effects on inflammatory diseases, including cancer and type 2 diabetes (7-9). Hence, the effects of oral LPS administration were not considered to correspond to the model of single LPS adipocyte stimulation.

Recently, repeated macrophage stimulation has been applied as an *in vitro* model for inducing tissue activating macrophage properties by oral LPS administration. While a single LPS stimulation of macrophages strongly induces the expression of inflammatory factors, repeated LPS stimulation induces phagocytosis of foreign substances, expression of anti-inflammatory factors, and tissue-protective factors. Additionally, this type of activation of macrophages is similar to the activation of tissue macrophages induced by oral LPS administration (10-15). These results show that the state of cells induced by repeated LPS stimulation is quite different from that of cells induced by a single stimulation. Therefore, if this response is adapted to adipocytes, repeated LPS adipocyte stimulation may show anti-inflammatory activities and elevated insulin signaling, unlike single stimulation.

In this study, for the first time, adipocytes were repeatedly stimulated with LPS to investigate whether their response is different from that following a single stimulation. 3T3-L1 adipocytes, the most widely used adipocyte line, were stimulated with low (1 ng/ml) or high (100 ng/ml) doses of LPS once or thrice, and the expression levels of inflammatory

and anti-inflammatory factors as well as insulin signaling-related factors were analyzed.

Materials and Methods

Cell culture and LPS treatment. Mouse embryo 3T3-L1 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The experimental design is shown in Figure 1. 3T3-L1 pre-adipocytes were grown in cultured in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented using 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) at 37˚C in 5% CO_2. To induce differentiation into mature adipocytes, 3T3-L1 pre-adipocytes were induced with differentiation media (DMEM with low glucose content supplemented with 10% fetal bovine serum, 2.5-μM dexamethasone (DEX), 0.5-mM 3-Isobutyl 1-methylxanthine (IBMX), and 10 μg/ml insulin (day 0 to day 2). At the beginning of day two, the medium was replenished with maturation media (DMEM with high glucose content supplemented with 10% fetal bovine serum and 10 μg/ml insulin) and kept at 37˚C in a humidified 5% CO_2 atmosphere. This medium was changed every two days until day eight. At this time, the cells showed the characteristics of mature adipocytes. On day eight, the medium was replenished with DMEM with high glucose content supplemented with 10% fetal bovine serum without insulin and 3T3-L1 mature adipocytes were treated with or without LPS [1 ng/ml or 100 ng/ml, purified LPS obtained from *Pantoea agglomerans* (Macrophi Inc., Kagawa, Japan)]. The concentration of 1 ng/ml LPS was defined as a "low-dose" and that of 100 ng/ml as a "high-dose control" as it has been reported that cytokines were induced by 1 ng/ml of LPS (11-13, 15), while LPS was generally used at 100 ng/ml to activate macrophages *in vitro*. For the repetitive treatment, cells received fresh medium containing LPS thrice every 24 h. For single treatment, cells received fresh medium without LPS for the first 48 h and then received a one-time dose of LPS in fresh medium. Samples were obtained after five hours to extract RNA or 24 h to measure cytokines in culture supernatants after the last LPS treatment.

Determination of cytokines using enzyme-linked immuno sorbent assay (ELISA). The culture supernatants were obtained 24 h after the last treatment of 3T3-L1 cells with LPS (1 ng/ml, or 100 ng/ml). The levels of IL-6 in the culture supernatants were measured using a commercial sandwich ELISA kit (BioLegend, San Diego, CA, USA), following the manufacturers' instructions. The levels of MCP-1 in the culture supernatants were measured using a commercial sandwich ELISA kit (Proteintech, Tokyo, Japan), according to the manufacturers' instructions. The levels of adiponectin in the culture supernatants were measured using a commercial sandwich ELISA kit (Otsuka Pharmaceutical, Tokyo, Japan), following the manufacturers' instructions.

Quantitative RT-PCR. RNA was acquired using the RNeasy mini kit (QIAGEN, Hilden, Germany), and cDNA was synthesized using reverse transcription using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan), following the manufacturer's instructions. A real-time PCR assay was performed using 5-μl cDNA as the template and 10-μl Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) on the Stratagene Mx 3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA). The primers are listed in Table I. The data were assessed based on the $2^{-\Delta\Delta Ct}$ method and normalized using *Gapdh* expression. The qPCR amplification was conducted with an activation step at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 s (denaturation) and 60˚C for 1 min (annealing), and a dissociation stage at 95˚C for 15 s, 60˚C for 30 s, and 95˚C for 15 s for each gene.

Statistical analysis. Statistical analysis was conducted using Ekuseru-Toukei 2012 (SSRI, Tokyo, Japan). The results are indicated as the mean±standard error (SE). The differences between the groups of mice were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test. Significance was set at $p<0.05$.

Results

Effect of repeated LPS stimulation on inflammation in 3T3-L1 adipocytes. To determine the effect of repeated LPS stimulation on inflammation in 3T3-L1 adipocytes, inflammatory and anti-inflammatory factor mRNA expression levels in adipocytes and protein levels in culture supernatants were measured. The mRNA expression levels of the inflammatory factors *Il6* and *Mcp1* were significantly elevated by single 1 ng/ml and 100 ng/ml LPS stimulation compared to the no stimulation group, but this increase was suppressed by repeated stimulation (Figure 2A and B). Additionally, IL-6 levels in the culture supernatant were significantly elevated by single 1 ng/ml and 100 ng/ml LPS stimulation compared to the no stimulation group, but this increase was suppressed through repeated stimulation (Figure 2C). Alternatively, the amount of MCP-1 in the culture supernatant was unaffected by 1 ng/ml LPS stimulation and was significantly elevated by single stimulation with 100 ng/ml LPS compared to the no stimulation group, but the amount of MCP-1 in the culture supernatant was unaffected by 100 ng/ml LPS repeated stimulation (Figure 2D).

The mRNA and protein expression of adiponectin (*Adipoq*), proliferator-activated receptor γ (*Pparγ*), and peroxisome proliferator-activated receptor gamma coactivator1 α (*Pgc1α*) were measured. Adipoq, Pparγ, and Pgc1α inhibit IL-6 and Mcp-1 expression and are involved in the anti-inflammatory effects of adipocytes. Adiponectin exhibited no change in mRNA expression and supernatant protein levels upon LPS stimulation (Figure 3A and D). mRNA expression of *Pparγ* was unaffected by single 1 ng/ml and 100 ng/ml LPS stimulation, but a significant increase or a trend toward an increase was observed with repeated stimulation (Figure 3B). *Pgc1α* mRNA expression indicated a trend toward a decrease or significant decrease with single 1 ng/ml and 100 ng/ml LPS stimulation compared to the no stimulation group, but this change was suppressed with repeated stimulation (Figure 3C).

These results show that repeated LPS stimulation, unlike single stimulation, inhibits the elevated expression of inflammatory factors and induces the expression of anti-inflammatory factors in adipocytes.

Effect of repeated LPS stimulation on insulin sensitivity of 3T3-L1 adipocytes. To determine the effect of repeated LPS stimulation on insulin sensitivity of 3T3-L1 adipocytes,

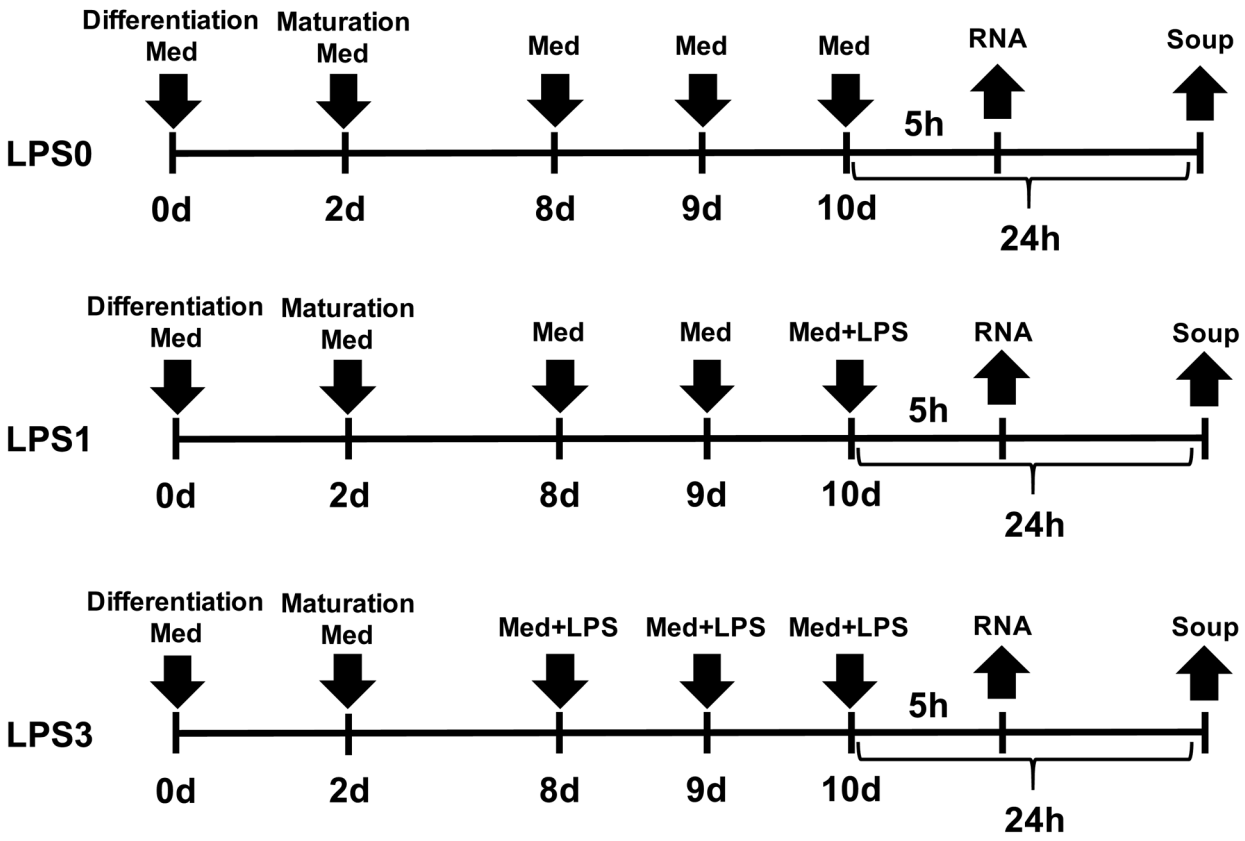

Figure 1. *Experimental design. LPS: Lipopolysaccharide; Med: medium; Sup: culture supernatants.*

Table I. *Primer pairs used for the quantitative RT-PCR analysis.*

Genbank ID	Target gene	Primer	Primer sequence (5'-3')
NM_009605	*Adipoq*	F	TGTTCCTCTTAATCCTGCCCA
		R	CCAACCTGCACAAGTTCCCTT
NM_001110208	*Akt2*	F	ACGTGGTGAATACATCAAGACC
		R	GCTACAGAGAAATTGTTCAGGGG
NM_001289726	*Gapdh*	F	CGACTTCAACAGCAACTCCCACTCTTCC
		R	TGGGTGGTCCAGGGTTTCTTACTCCTT
NM_001359114	*Glut4*	F	GTAACTTCATTGTCGGCATGG
		R	AGCTGAGATCTGGTCAAACG
NM_031168	*Il6*	F	CCAGAGATACAAAGAAATGATGG
		R	ACTCCAGAAGACCAGAGGAAAT
NM_010568	*Ir*	F	TTTGTCATGGATGGAGGCTA
		R	CCTCATCTTGGGGTTGAACT
NM_010570	*Irs1*	F	CCATGAGCGATGAGTTTCGC
		R	GCAGTGATGCTCTCAGTTCG
NM_011333	*Mcp1*	F	AACTGCATCTGCCCTAAGGT
		R	ACTGTCACACTGGTCACTCC
NM_011146	*Pparγ*	F	CCATTCTGGCCCACCAAC
		R	AATGCGAGTGGTCTTCCATCA
NM_008904	*Pgc1α*	F	GCGAACCTTAAGTGTGGAAC
		R	CACCACGGTCTTGCAAGAGG

Adipoq: Adiponectin; *Akt2*: thymoma viral proto oncogene 2; *Gapdh*: glyceraldehyde 3 phosphate dehydrogenase; *Glut4*: glucose transporter type 4; *Il6*: interleukin 6; *Ir*: insulin receptor; *Irs1*: insulin receptor substrate 1; *Mcp1*: monocyte chemotactic protein 1; *Pparγ*: peroxisome proliferator activated receptor gamma; *Pgc1α*: peroxisome proliferator activated receptor gamma coactivator 1 alpha.

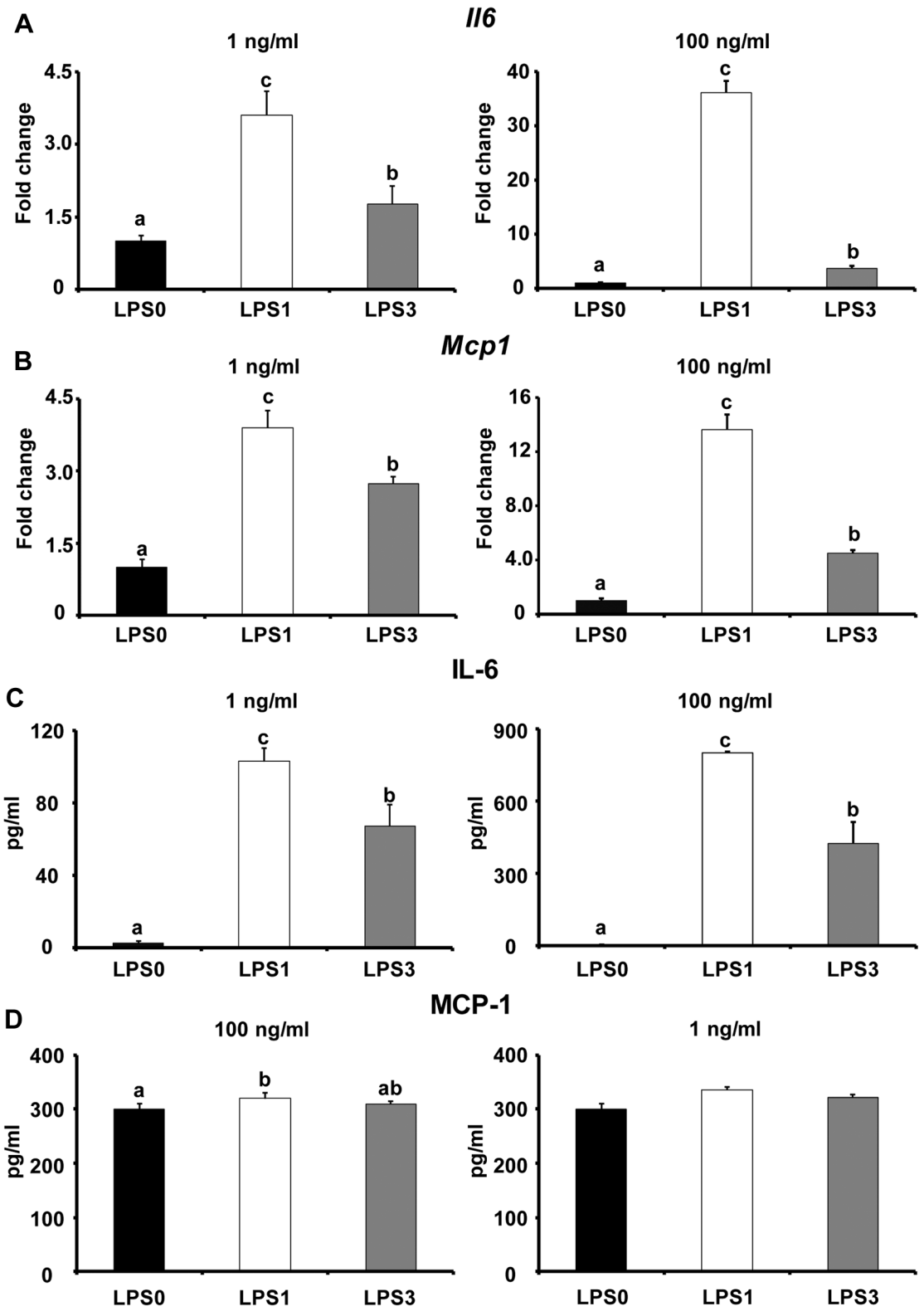

Figure 2. *Analysis of mRNA expression of interleukin-6 (Il6) (A) and monocyte chemotactic protein 1 (Mcp1) (B) in 3T3-L1 cells treated with 1 ng/ml, or 100 ng/ml lipopolysaccharide (LPS) once or thrice and protein levels of IL-6 (C) and MCP-1 (D) in the culture supernatant of 3T3-L1 cells treated with 1 ng/ml, or 100 ng/ml LPS once or thrice. LPS0: no treatment with LPS; LPS1: single treatment with LPS; LPS3: thrice treatment using LPS every 24 h. Values are presented as mean±SE, n=3. Different letters indicate significant differences, p<0.05.*

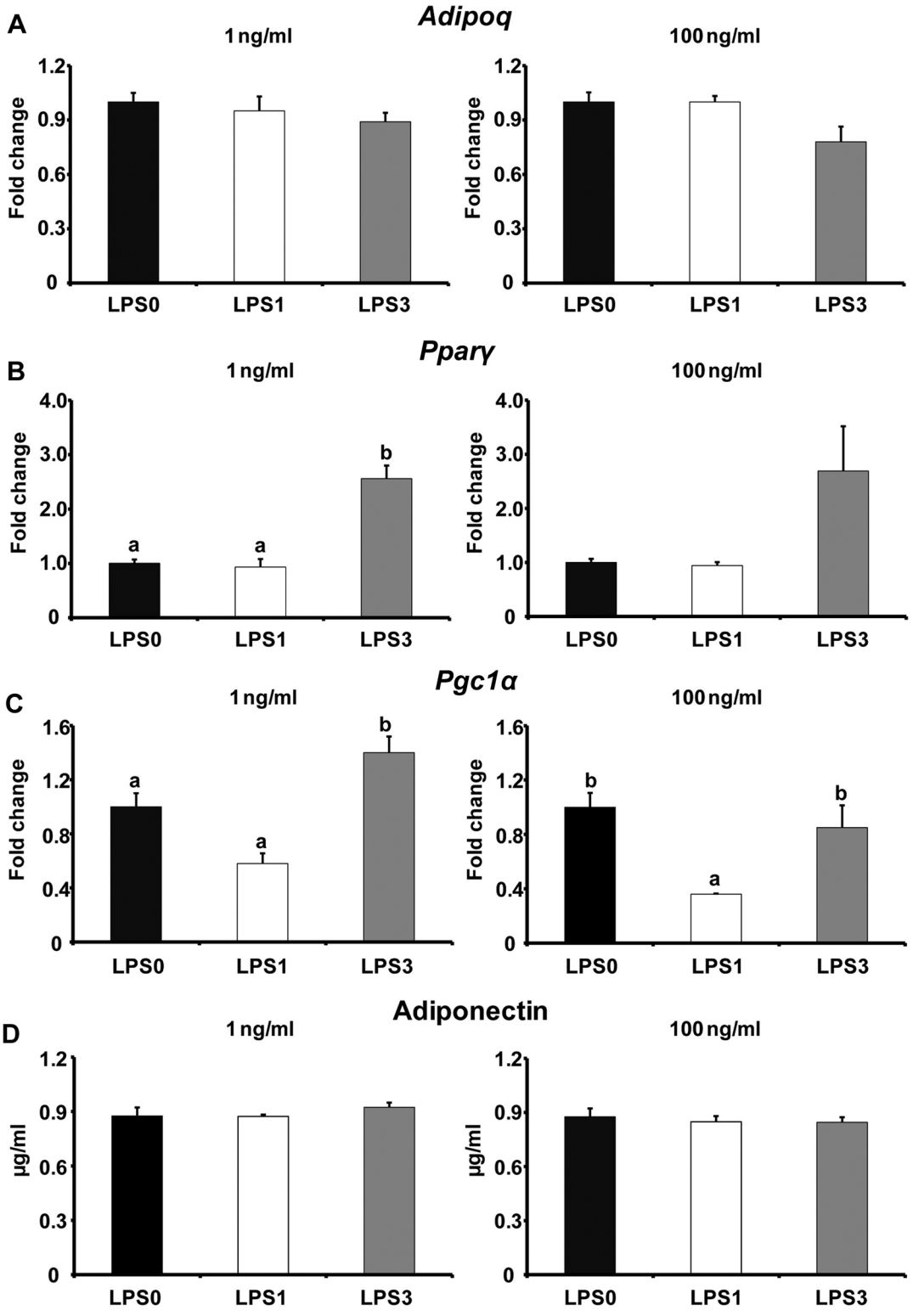

Figure 3. *Analysis of mRNA expression of adiponectin (Adipoq) (A), proliferator-activated receptor γ (Pparγ) (B), and peroxisome proliferator-activated receptor gamma coactivator1 α (Pgc1α) (C) in 3T3-L1 cells treated with 1 ng/ml, or 100 ng/ml lipopolysaccharide (LPS) once or thrice and protein levels of adiponectin (D) in the culture supernatant of 3T3-L1 cells treated with 1 ng/ml, or 100 ng/ml LPS once or thrice. LPS0: no treatment with LPS; LPS1: single treatment using LPS; LPS3: thrice treatment using LPS every 24 h. Values are presented as mean±SE, n=3. Different letters indicate significant differences, p<0.05.*

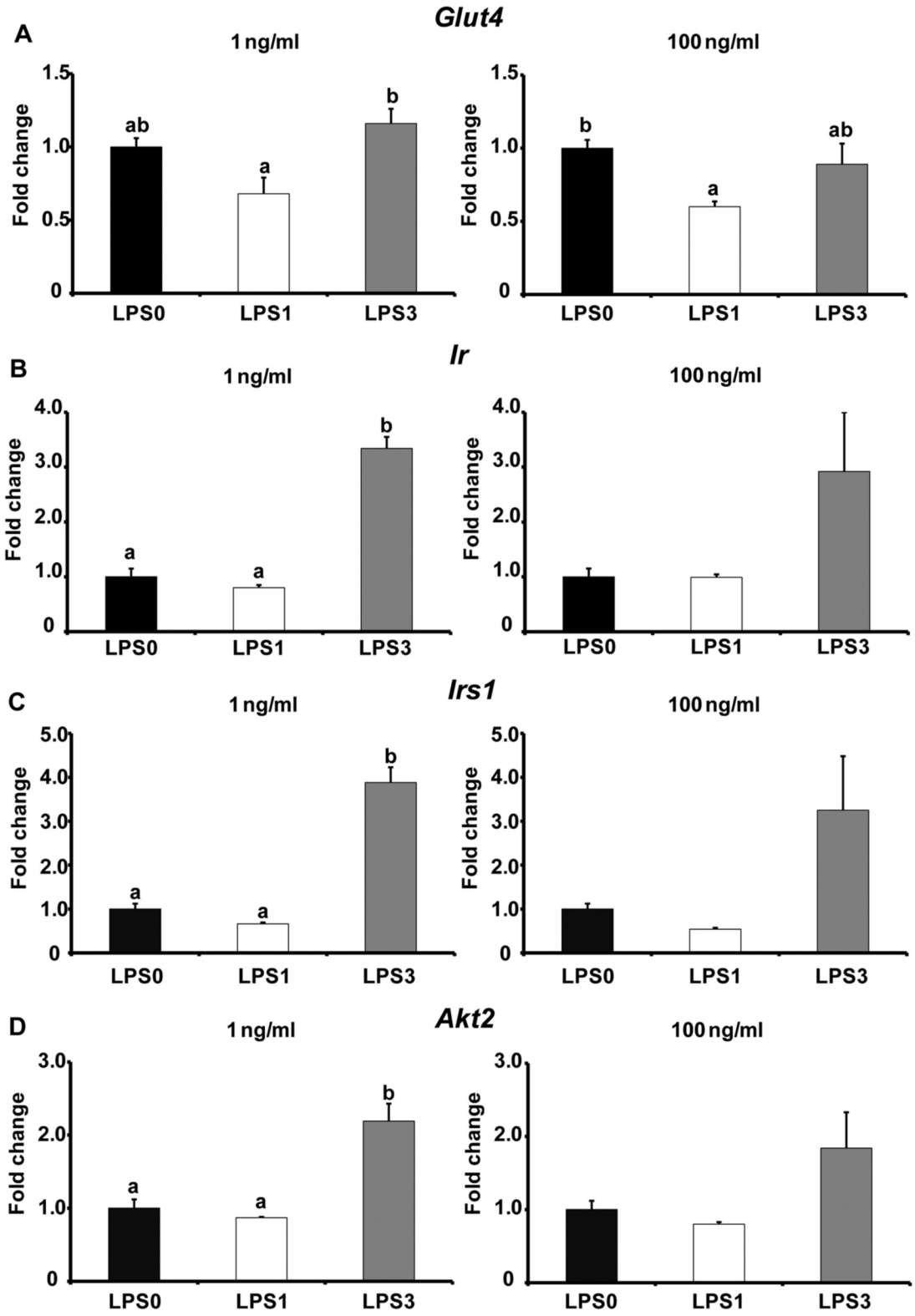

Figure 4. *Analysis of mRNA expression of glucose transporter type 4 (Glut4) (A), insulin receptor (Ir) (B), insulin receptor substrate 1 (Irs1) (C), and thymoma viral proto-oncogene 2 (Akt2) (D) in 3T3-L1 cells treated with 1 ng/ml, or 100 ng/ml lipopolysaccharide (LPS) once or thrice. LPS0: no treatment with LPS; LPS1: single treatment using LPS; LPS3: thrice treatment using LPS every 24 h. Values are presented as mean±SE, n=3. Different letters indicate significant differences, p<0.05.*

mRNA expression levels of insulin sensitivity-related molecules were measured. The mRNA expression level of *Glut4*, which transports glucose in the cell, exhibited a reduction trend or significant reduction by single LPS stimulation at 1 ng/ml and 100 ng/ml, but this change was suppressed by repeated LPS stimulation (Figure 4A). The expression levels of insulin receptor (*Ir*), Insulin receptor substrate 1 (*Irs1*), and thymoma viral proto-oncogene 2 (*Akt2*), which are involved in insulin signaling, were unaffected by single LPS stimulation at 1 ng/ml and 100 ng/ml but exhibited a significant increase or increasing trend by repeated LPS stimulation compared to no stimulation or single stimulation (Figure 4B, C, and D).

These results show that LPS repetitive stimulation, unlike single stimulation, increases the expression of insulin sensitivity-related factors in adipocytes.

Discussion

Previously, it was found that repeated LPS stimulation of macrophages expressing the LPS receptor Toll-like receptor 4 (TLR4) induced features, which are distinct from the characteristics induced by single LPS stimulation and these features were similar to those of tissue macrophages induced by oral LPS administration (10-15). In this study, we examined whether repeated LPS stimulation of adipocytes, which also express TLR4, induces features different from single LPS stimulation.

Single LPS stimulation of adipocytes induced increased expression of inflammatory factors, such as IL-6 and Mcp-1, which are involved in chronic inflammation in adipocytes (1, 2, 4) (Figure 2). These results agreed with previous reports, which performed single LPS adipocyte stimulation (4-6). Therefore, the model of LPS adipocyte stimulation used in this study is a suitable model for evaluating LPS effects on adipocytes.

Unlike single LPS stimulation, repeated LPS stimulation inhibited the elevated expression of inflammatory factors such as IL-6 and Mcp-1. Furthermore, the expression of Pparγ and Pgc1α, involved in the anti-inflammatory response of adipocytes and were not upregulated by single LPS stimulation, increased (Figure 3B and C). These factors are involved in the reduced expression of IL-6 and Mcp-1 (16, 17). Our previous studies also indicated that a single LPS stimulation of macrophages strongly induced the expression of inflammatory factors, such as IL-6, IL-1β, and TNFα, but repeated LPS stimulation suppressed the expression of these factors and induced the expression of anti-inflammatory factors, such as IL-10 (11-13). These results show for the first time that repeated LPS stimulation induces anti-inflammatory properties in adipocytes, which are different from those induced by a single LPS stimulus.

A single LPS adipocyte stimulation induces the expression of inflammatory factors and decreases the expression of insulin signaling-related factors. In this study, as in previous reports, a single LPS stimulation decreased Glut4 expression, which transports glucose into the cell, suggesting that insulin signaling in adipocytes was reduced (5). However, repeated LPS stimulation reversed the reduction in Glut4 expression. Furthermore, the expression levels of Ir, Irs1, and Akt2 were unaffected by a single LPS stimulation, but were increased through repeated LPS stimulation (Figure 4). Induction of Glut4 expression in adipocytes via Ir, Irs1, and Akt2 expression promotes glucose uptake from the blood into adipocytes. This mechanism prevents insulin resistance and glucose intolerance and prevents type 2 diabetes (18-23). Based on the above, the phenotype of adipocytes following repeated LPS stimulation may reflect the phenotype that prevents type 2 diabetes.

It has been thought that LPS is an inducer of type 2 diabetes because a reduction in insulin signaling is induced following single LPS adipocyte stimulation (4-6). However, quite different from conventional thinking, the phenotype that is acquired by adipocytes following repeated LPS stimulation does not support the hypothesis that LPS induces type 2 diabetes. These results overturn the previous perception on the effect of LPS on the development of type 2 diabetes.

In this study, LPS adipocyte stimulation was performed at a low-dose (1 ng/ml) and a high-dose (100 ng/ml). Stimulation with 100 ng/ml–10 μg/ml LPS is usually employed in *in vitro* models. Previously, macrophages were repeatedly stimulated with low (1 ng/ml) and high (100 ng/ml) doses of LPS, and it was observed that they acquired different properties. Macrophages induced by repeated low-dose LPS stimulation were more reflective of the state of tissue macrophages induced by oral LPS administration (12-14). Therefore, as a model of the oral LPS administration, the effect of stimulation with low-dose LPS (1 ng/ml) was compared to that of stimulation with conventional high-dose (100 ng/ml) LPS. The results indicated that the expression pattern of inflammatory factors, anti-inflammatory factors, and insulin signaling-related factors in adipocytes was not different between 1 ng/ml and 100 ng/ml LPS stimulation. This suggested that adipocytes, unlike macrophages, did not acquire different properties because of LPS-stimulated doses.

This study indicates that repeated LPS adipocyte stimulation suppressed the upregulation of inflammatory factors (IL-6, Mcp-1) and increased the expression of anti-inflammatory factors (Pparγ, Pgc1α) and insulin signaling-related factors (Glut4, Ir, Irs1, Akt2). This indicated that repeated LPS stimulation induced properties that are quite different from those induced by single LPS stimulation, such as elevated inflammatory factors and reduced insulin signaling-related factors. Until now, the fact that oral LPS administration induces anti-inflammatory properties has remained unclear because this effect is inconsistent with single LPS stimulation *in vitro*, but the use of repetitive LPS

stimulation could contribute to further studies. Adipocytes exhibiting anti-inflammatory effects and the induction of insulin signaling by repeated LPS stimulation are observed when type 2 diabetes is prevented (18-23). Furthermore, in previous mice studies, it was observed that oral LPS administration suppressed high-fat diet-induced elevated expression of IL-6 and Mcp-1 in the blood, and these mice were also resistant to type 2 diabetes (8, 9). In conclusion, although it is necessary to compare adipocytes induced by repeated LPS stimulation with those induced by oral LPS administration, adipocytes induced by repeated LPS stimulation may serve as an *in vitro* model of adipocytes induced by oral LPS administration and may be used to analyze the preventive LPS effect on type 2 diabetes mellitus.

Conflicts of Interest

KY, MY, HI and GS are employed by the Control of Innate Immunity, Technology Research Association. HI, CK, and GS are employed by Macrophi Inc. This does not alter our adherence to journal policies on sharing data and materials.

Authors' Contributions

KY, HI, CK and GS conceptualized the study and coordinated the experiments. KY and MY performed the experiments and KY performed data curation and formal analysis. HI and GS acquired the funding and administrated the project.

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