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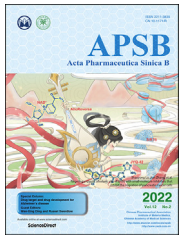

LETTER TO THE EDITOR

Peptidoglycan inhibits beigeing of adipose tissue

KEY WORDS

Beige adipocytes;
Brown adipocytes;
Obesity;
Metabolic inflammation

To the Editor:

Obesity, an epidemic metabolic disease characterized by excessive fat accumulation, causes a significant economic burden on families and the society. The discovery of beige adipocytes has provided us with a brand new approach for the intervention of obesity[1]. Beige adipocytes increase energy expenditure and improve the balance of glucose and lipid metabolism. Reduction of beige adipocytes is a hallmark of obesity. Mechanism underlying the decrement of beigeing remains largely unknown.

Intestinal microflora is strongly associated with white adipose tissue beigeing. Removal of intestinal microflora either with antibiotic treatment or in germ-free mice can effectively promote beigeing of subcutaneous and visceral adipose tissue[2]. However, it is still unclear how the intestinal microflora influences white adipose tissue beigeing. Previous studies have suggested that bacterial products may affect the thermogenesis of white and brown adipose tissue[3]. For example, lipopolysaccharides (LPS) from Gram-negative bacteria have been reported to suppress adaptive thermogenesis[4]. However, studies have shown an increase in the ratio of Gram-positive firmicutes to Gram-negative bacteroidetes[5]. These observations suggest that products of intestinal Gram-positive bacterial may also contribute to modulation of beigeing.

Peptidoglycan (PGN) is a unique and essential structural element in the cell wall of Gram-positive bacteria. It is embedded in a relatively thick cell wall with other polymers, such as lipoteichoic acids (LTAs). Our previous study suggests that PGN plays an important role in insulin resistance and metabolic inflammation[6]. Whether PGN affects the beigeing of adipose tissue remains unclear. PGNs from diverse bacteria function through Toll-like receptor (TLR) 2 to activate multiple signaling such as nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK). As a critical molecule in innate immune response[7], TLR2 may be involved in diet-induced metabolic syndrome. TLR2 deficiency improves insulin sensitivity, beta cell dysfunction and hepatic insulin signaling in mice[8]. It remains unclear whether the beneficial effects of TLR2 deficiency occur indirectly by suppressing inflammatory cells in metabolic tissues or directly by acting on parenchymal cells.

Here we report that PGN suppresses the white adipose tissue beigeing. This occurs through induction of inflammation in adipose tissue by promoting macrophage M1 polarization. In addition, PGN directly activates TLR2 receptor on adipocytes to suppress beigeing. Intervention of PGN-TLR2 signaling may thus provide a potential strategy for treatment of obesity.

1. PGN inhibits beigeing of white adipose tissue in mice

To determine whether PGN contributes to the dysfunction of lipid metabolism, we first examined the plasma concentration of PGN in diet-induced obesity (DIO) mice. As shown in Fig. 1A, plasma levels of PGN were significantly increased compared with lean mice fed normal chow diet (NCD), indicating a close relation between PGN and obesity. This concept was supported by our previous observation showing that PGN may induce nonalcoholic fatty liver disease[6]. We then examined whether PGN alters the beigeing of white adipose tissue in mice. PGN was injected intraperitoneally at a dose of 4 mg/kg/day for 8 days at room temperature. This dose of PGN increased its plasma concentration to the level comparable to DIO mice (Fig. 1B). Mice were then transferred to 4 °C cold room to induce beigeing and received the same dose of PGN for another 2 days. Although PGN demonstrates no significant effect on the body weight and food intake (Supporting Information Fig. S1A and S1B), both the plasma triglycerides and cholesterol showed a moderate increase (Fig. S1C and S1D).

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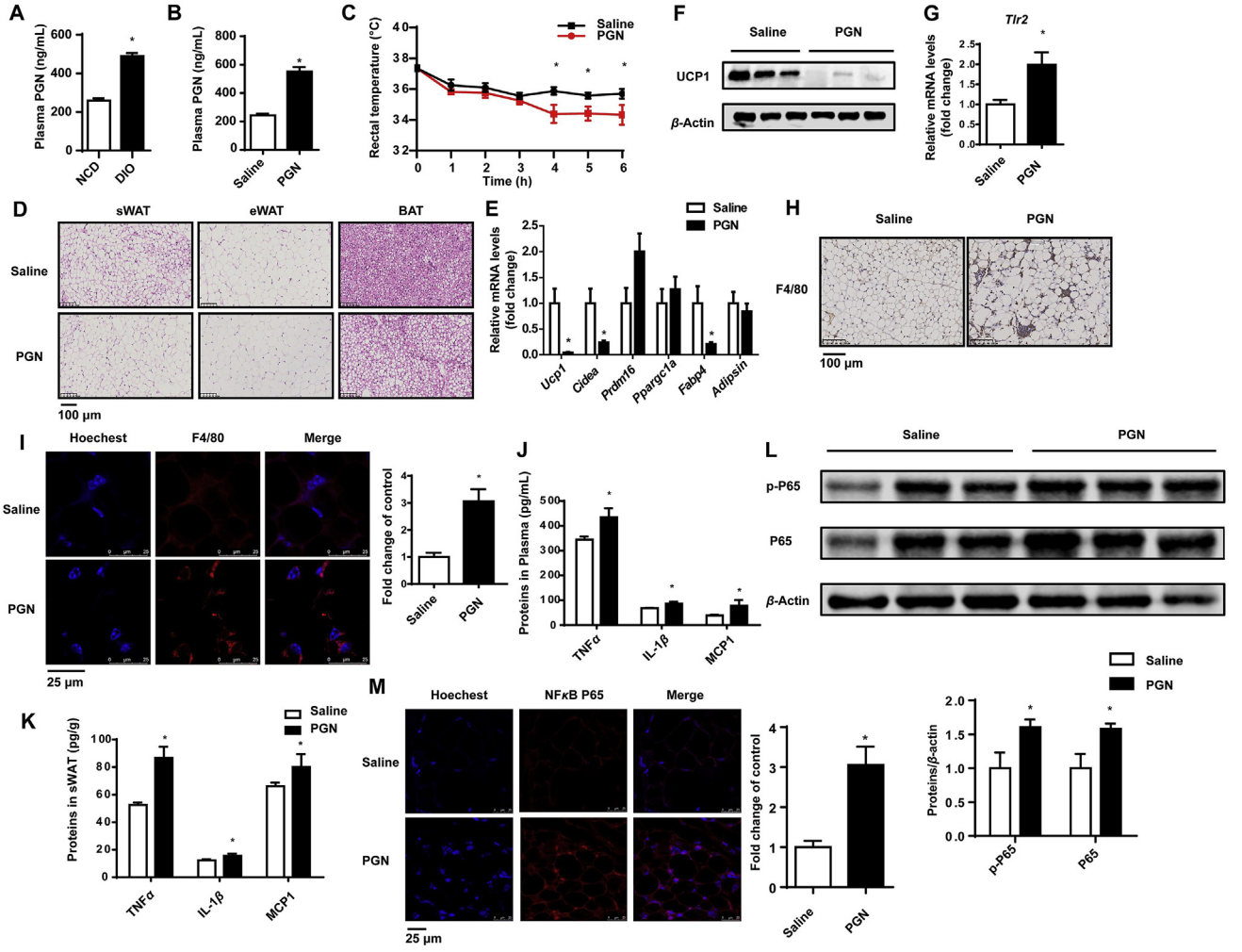

Figure 1 PGN inhibits sWAT beigeing and promotes macrophage M1 polarization in mice. (A) Plasma levels of PGN. Lean mice were fed NCD. Obese mice (DIO) were induced with 60% high-fat diet for 12 weeks. NCD group, $n = 5$; DIO group, $n = 7$. (B) Plasma levels of PGN. PGN was administrated daily at a dose of 4 mg/kg/day for a consecutive 10 days *via* intraperitoneal injection. (C) Rectal core body temperature. Mice were exposed to 4 °C cold temperature. Saline group, $n = 5$; PGN group, $n = 6$. $^*P < 0.05$ by unpaired Student's *t* test. (D) H&E staining in sWAT, eWAT and BAT. Scale bar = 100 μm. (E) mRNA levels of thermogenic and adipogenic genes in sWAT. Quantitative RT-PCR results were normalized to the geomean of *Hprt*, *Tbp* and *Mrpl32*. Saline group, $n = 7$; PGN group, $n = 9$. (F) Protein levels of UCP1. β-Actin was used as the loading control. (G) mRNA expression levels of *Tlr2* in sWAT. Mice were treated with PGN or saline as indicated above. (H) F4/80 immunoreactivity in sWAT. Scale bar = 100 μm. (I) Confocal microscopy images for F4/80 immunofluorescence. Nuclei were stained with DAPI (blue). Scale bar = 25 μm. Signal intensity was quantified using Image J. (J) Levels of pro-inflammatory cytokines in plasma. Saline group, $n = 7$; PGN group, $n = 9$. (K) Levels of pro-inflammatory cytokines in sWAT. Saline group, $n = 7$; PGN group, $n = 9$. (L) Protein levels of P65, phospho-P65 (p-P65) detected by Western blotting. β-Actin was used as the loading control. Relative protein signal intensity was quantified using Image J software. (M) Confocal microscopic images for P65. Nuclei were stained with DAPI (blue). Scale bar = 25 μm. Signal intensity was quantified using Image J. Data are presented as the mean ± SEM, $n = 5$ per group unless indicated otherwise. $^*P < 0.05$ by unpaired Student's *t* test.

Consistently, our previous studies[6] showed that PGN promotes the development of steatohepatitis in lean mice, which is associated with significantly impaired glucose tolerance and insulin sensitivity. These results indicate that PGN may induce metabolic dysfunction in lean mice, which may be clinically relevant to type 2 diabetes and nonalcoholic steatohepatitis (NASH) in lean objects. Importantly, cold exposure induced a more significant reduction in core body temperatures in mice treated with PGN (Fig. 1C). Analysis of adipose tissues by H&E staining revealed a major change in sWAT and BAT. Cold exposure stimulated the formation of beige adipocytes with multilocular lipid droplets in sWAT. The cold-induced beigeing of sWAT was significantly impaired by PGN (Fig. 1D). This alteration was associated with a significant increment in the size of adipocytes (Fig. 1D). Further, PGN significantly decreased the mRNA levels of thermogenic genes such as uncoupling protein 1 (*Ucp1*), cell death-inducing DNA fragmentation factor alpha subunit-like effector A (*Cidea*) in sWAT (Fig. 1E). Consistently, protein levels of UCP1 were markedly reduced by PGN evidenced by Western blotting (Fig. 1F). Besides, PGN increased lipid accumulation and the size of adipocytes in BAT (Fig. 1D). Taken together, these results indicate that PGN inhibits beigeing of sWAT and induces BAT whitening.

2. PGN inhibits sWAT beigeing by promoting macrophage M1 polarization

PGN has been demonstrated to trigger innate inflammation by activation of its receptor TLR2. We thus analyzed the TLR2 levels in sWAT. As shown in Fig. 1G, mRNA levels of *Tlr2* gene in sWAT were markedly increased by PGN. Since abundant expression of TLR2 in macrophages has been reported, we next examined the macrophages in sWAT. F4/80 positive macrophages and abundance of crown-like structure in sWAT were significantly increased by PGN as evidenced by both immunohistochemical (Fig. 1H) and immunofluorescent staining (Fig. 1I). Protein levels of inflammatory factors relevant to M1 macrophages, TNFα, IL-1β and MCP1 in plasma (Fig. 1J) and sWAT (Fig. 1K) were significantly increased compared with control mice. These alterations were associated with an increment in P65, phospho-P65 (Fig. 1L) and nuclear translocation of P65 (Fig. 1M). These observations suggest that PGN promotes macrophage M1 polarization in sWAT which may contribute to the suppression of beigeing.

To demonstrate this concept, we used the co-culture of Stromal Vascular Fraction (SVF) cells isolated from sWAT and macrophages. SVF cells were successfully induced to differentiate into beige cells evidenced by abundant expression of UCP1 protein (data not shown). Treatment of cultured Raw264.7 cells, a macrophage cell line, with PGN significantly increased the M1 polarization. mRNA levels of M1 marker genes including *Tnfα*, *Mcp1*, nitric oxide synthase 2, inducible (*Nos2*) were markedly increased (Fig. 2A). Levels of M2 marker genes *Mgl-1* and *Arg-1* were significantly reduced (Fig. 2A). Conditional medium from PGN-induced M1 macrophages significantly suppressed the differentiation of SVF cells into beige adipocytes as evidenced by the reduction of thermogenic genes *Ucp1* and *Cidea* (Fig. 2B). Similar results were observed for LPS (Fig. 2B).

3. PGN inhibits beigeing of SVF cells through TLR2

Since adipocytes express PGN receptor, TLR2, we next investigated whether PGN has direct effects on the differentiation of SVF

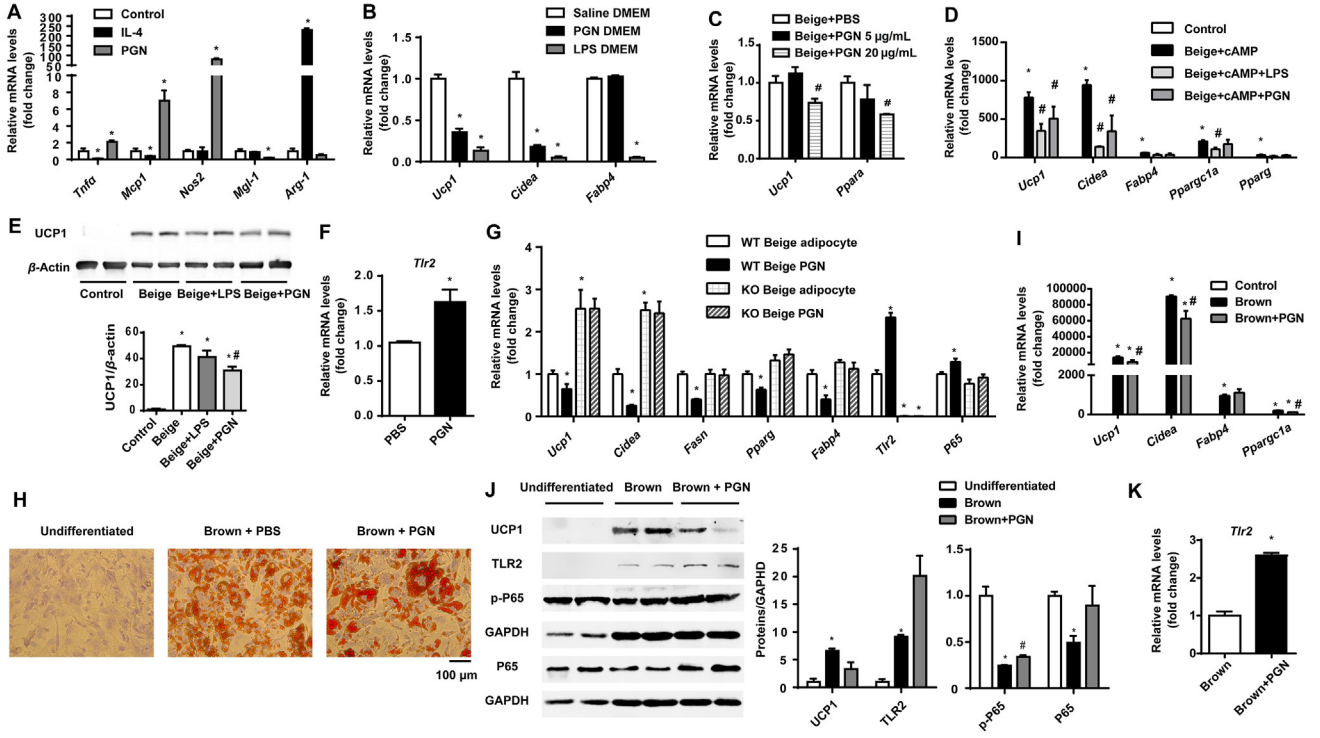

Figure 2 PGN inhibits beigeing of SVF cell directly and/or indirectly by macrophage. (A) mRNA levels of M1 and M2 macrophage marker genes of Raw264.7 cells treated with PBS, IL-4 (10 ng/mL) or PGN (20 μg/mL) for 6 h ($n = 4$ for each group). (B) mRNA levels of thermogenic and lipogenic genes of beiged SVF cells treated with conditional medium from Raw264.7 treated with saline, PGN (20 μg/mL) or LPS (10 ng/mL) for 24 h ($n = 3$ for each group). (C) mRNA levels of *Ucp1* and *Pparα* in beiged SVF cells treated with or without PGN. (D) mRNA levels of thermogenic genes in beiged SVF cells treated with LPS (10 ng/mL) or PGN (20 μg/mL) for 24 h. Cells were stimulated with cAMP at 0.5 μmol/L for 12 h. Undifferentiated SVF cells was used as a control. $^{*}P < 0.05$ *vs*. Control; $^{\#}P < 0.05$ *vs*. Beige+cAMP. (E) Protein levels of UCP1. β-Actin was used as the loading control. Relative protein signal intensity was quantified using Image J software. (F) mRNA levels of *Tlr2* in beige adipocytes treated with PBS or PGN. (G) mRNA expression levels of thermogenic genes, adipogenic genes, *Tlr2* and *P65* in differentiated beiged SVF cells treated with saline or PGN. SVF cells were isolated from AdioQ-TLR2$^{-/-}$ mice (KO) or wild type littermates (WT). Cells were differentiated for 7 days under a beige adipogenic condition and treated with PGN (20 μg/mL) every other day during the differentiation. (H) Oil red O staining of undifferentiated brown preadipocytes, brown adipocytes treated with PBS or PGN (20 μg/mL). Scale bar = 100 μm. (I) mRNA levels of genes relevant to thermogenesis and lipogenesis. $^{*}P < 0.05$ *vs*. Undifferentiated brown preadipocytes; $^{\#}P < 0.05$ *vs*. brown adipocytes by two-way ANOVA. (J) Protein levels of UCP1, TLR2, NF-κB P65 and phospho-P65 (p-P65). GAPDH was used as the loading control. Relative protein signal intensity was quantified using Image J software. (K) mRNA levels of *Tlr2* in brown adipocytes treated with PBS or PGN. $^{*}P < 0.05$ *vs*. brown adipocytes by unpaired Student's *t* test. Data are presented as the mean ± SEM, $n = 3$–6. $^{*}P < 0.05$ by unpaired Student's *t* test or two-way ANOVA. mRNA expression was normalized to the geomean of *Hprt*, *Tbp* and *Mrpl32*.

cells into beige adipocytes. Previous studies[6] have reported that 20 μg/mL is the efficient dose of PGN on cultured cells. Consistently, PGN at a concentration of 20 μg/mL significantly suppressed the differentiation of cultured SVF cells into beige adipocytes (Fig. 2C). PGN at this dose also attenuated the cAMP-induced up-regulation of beigeing in cultured SVF cells. mRNA levels of *Ucp1*, *Cidea* were significantly reduced whereas fatty acid binding protein 4 (*Fabp4*), peroxisome proliferator activated receptor gamma (*Pparg*) remained unchanged (Fig. 2D). Protein levels of UCP1 were consistently down-regulated (Fig. 2E). Interestingly, PGN significantly increased mRNA levels of *Tlr2* in differentiated beige adipocytes (Fig. 2F). We thus explored whether PGN functions through TLR2 to inhibit beigeing. PGN demonstrated no effect on the suppression of beigeing differentiation in SVF cells with deficiency of TLR2 derived from AdioQ-TLR2$^{-/-}$ mice (Fig. 2G). In these cells, PGN also failed to inhibit the expression of *Ucp1* and *Cidea*, as well as to increase levels of *P65* (Fig. 2G). All these observations indicate that PGN suppressed the beigeing of sWAT by its direct activation of TLR2 on adiponectin positive adipocytes.

Besides, PGN significantly suppressed the differentiation of brown preadipocytes isolated from the neonatal mice as evidenced by oil red O staining (Fig. 2H). Expression of brown adipocyte marker genes were substantially attenuated by PGN as evidenced by the significant decrement in mRNA levels of *Ucp1*, *Cidea*, and peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (*Ppargc1a*) (Fig. 2I) and the protein levels of UCP1 (Fig. 2J). Moreover, PGN also significantly increased mRNA levels of *Tlr2* and protein levels of TLR2, NF-κB P65 and phospho-NF-κB P65 in differentiated brown adipocytes (Fig. 2J and K).

Based on these observations, we speculate that PGN inhibits the white adipose tissue beigeing by TLR2−NF-κB signaling. Further study should examine the distinct effect of different PGNs as molecular structure of peptidoglycan varies greatly dependent on individual type of gut bacteria. Second, the metabolic phenotype of TLR2 deficiency should be explored using transgenic mice with adipocyte and macrophage specific deletion of this receptor. Third, in addition to the NF-κB pathway, the JNK (c-Jun N-terminal kinase) pathway should be explored since this signaling pathway inhibits mitochondrial function through phosphorylation of BCL2[9,10].

In conclusion, our studies show that PGN activates TLR2−NF-κB signaling directly in adipocytes and/or indirectly in macrophages to inhibit the white adipose tissue beigeing. The activity of NF-κB remains to be tested by experiment in the inhibition of beigeing. PGN may serve as a critical linker between the intestinal bacteria and adipose tissue in the control of beigeing. Targeting PGN−TLR2 may provide a novel strategy for the prevention and treatment of obesity.

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Author contributions

Weizhen Zhang, Hong Chen designed the study; Hong Chen performed the experiments and analyzed the data; Lijun Sun and Lu Feng helped with some experiments. Michael Mulholland provided expertise; Hong Chen wrote the original draft; Weizhen Zhang reviewed and edited the manuscript; Weizhen Zhang and Yue Yin obtained funding and supervised the study. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no competing interests.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2021.11.015.

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Hong Chen[a], Lijun Sun[a], Lu Feng[a], Michael Mulholland[b], Weizhen Zhang[a,b,*], Yue Yin[a,*]

[a]*Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Key Laboratory of Molecular Cardiovascular Science, Ministry of Education, Peking University, Beijing 100191, China*

[b]*Department of Surgery, University of Michigan Medical Center, Ann Arbor, MI 48109-0346, USA*

[*]Corresponding authors. Tel.: +86 10 82802183, +86 10 82802514.

E-mail addresses: weizhenzhang@bjmu.edu.cn (Weizhen Zhang), yueyin@bjmu.edu.cn (Yue Yin)

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