

Lipopolysaccharide binding triggers insulin inactivation and HDL-dependent clearance

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Article

Keywords: insulin, lipopolysaccharide, insulin resistance, type 2 diabetes

Posted Date: August 25th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10599076/v1>

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Additional Declarations: There is **NO** Competing Interest.

Abstract

The canonical pathway for lipopolysaccharide (LPS)-induced insulin resistance is that LPS activates innate immune responses and triggers the secretion of pro-inflammatory cytokines to impair insulin signaling. We now report a rapid, inflammation-independent mechanism underlying LPS-mediated insulin signaling disruption. Mechanistically, LPS binds circulating insulin via hydrophobic interactions, and this physical interaction disrupts insulin-receptor engagement while suppressing downstream metabolic signaling cascades. This insulin dysfunction was induced by lipid A and it preceded the robust pro-inflammatory cytokine production. Furthermore, we identify a homeostatic clearance axis wherein LPS–insulin complexes can be sequestered by high-density lipoprotein (HDL) and eliminated via the SR-B1-dependent pathway. Therefore, our findings identify direct insulin-LPS binding as a novel mechanism underlying LPS-induced insulin resistance in type 2 diabetic mellitus (T2DM), complementing the classical inflammation-mediated pathway.

Introduction

T2DM, which constitutes the majority (90–95%) of all diabetes cases¹, is a progressive disease characterized by chronic hyperglycemia arising from defective insulin secretion, impaired insulin action, or both. Despite remarkable advances in glycemic management and pharmacotherapy, the rising prevalence of T2DM, coupled with incomplete remission and persistent complications, underscores that the mechanistic understanding of disease initiation and progression remains incomplete.

Lipopolysaccharide (LPS), the major structural form of endotoxin, is a crucial component of the outer membrane of gram-negative bacterium, and serves as one of their most pivotal virulence factors. While LPS is abundant in the healthy human gut microenvironment and modern dietary patterns, only a negligible amount translocates into the systemic circulation, with the vast majority being rapidly neutralized by host defense mechanisms^{2,3}. The pathological condition termed “metabolic endotoxemia” is precipitated by intestinal barrier dysfunction (commonly referred to as “leaky gut”), which is characterized by increased intestinal permeability and predominantly observed in individuals with obesity or diabetes mellitus^{4,5}. This barrier impairment facilitates the translocation of LPS across the intestinal epithelium, resulting in a 2–3 folds elevation in circulating LPS concentrations. Besides, T2DM patients are vulnerable to infections, among which gram-negative bacteria account for approximately 25% of cases⁶. Cumulative evidence indicates that prolonged exposure to endotoxemia is closely associated with the pathogenesis of T2DM, however, the precise mechanisms remain incompletely elucidated.

Current consensus mechanistic models suggest that LPS activates the host immune system, triggering the release of pro-inflammatory cytokines, including tumor necrosis factor (TNF) and multiple interleukins. These inflammatory mediators impair canonical insulin signal transduction by modulating the expression and phosphorylation dynamics of core insulin-signaling effector proteins^{7–10}. Persistent inflammatory injury further drives progressive pancreatic β -cell dysfunction, which in turn forms a self-

potentiating pathogenic loop to aggravate systemic insulin resistance^{11,12}. Update, no definite evidence has been established to confirm a direct causal association between LPS and insulin resistance. Here, we reveal that LPS directly binds insulin to trigger its functional inactivation, and potentiates lipoprotein-dependent insulin clearance.

Results

Insulin binds to LPS

In our earlier work, we found that LPS promoted insulin amyloid formation *in vitro*, which in turn inactivates insulin signal transduction¹³. This observation drove us to explore whether a direct interaction occurs between LPS and insulin. Given its amphiphilic nature, LPS tends to aggregate into supramolecular micelles in aqueous environment. Upon entering the plasma, specific LPS-binding molecules, including LBP¹⁴, secretoglobin (SCGB)3A2¹⁵, and caspase-4¹⁶, disassemble LPS micelles into monomers, which is essential for the recognition and clearance of LPS by immune cells. To investigate the potential of insulin as an LPS binding molecule, we examined LPS aggregation states using native agarose gel electrophoresis (NAGE), where LPS migration is dictated by both molecular charge and size¹⁷. Following electrophoretic separation, LPS bands were visualized via negative staining with imidazole and zinc. As illustrated in Fig. 1a, LPS alone displayed highly restricted migration, remaining largely confined around the electrode. Strikingly, insulin behaved analogously to that of lysozyme¹⁸, disrupting LPS aggregates and facilitating their electrophoretic migration in a dose-dependent manner (Fig. 1b). Furthermore, insulin disaggregated LPS derived from distinct genera, namely *E. coli* O111:B4, *E. coli* O55:B5, and *Salmonella Enteritidis*, which harbor conserved lipid A moieties but differ in their core oligosaccharides and O-antigens (Fig. 1c). We next employed dynamic light scattering (DLS) to measure the hydrodynamic diameter of LPS micelles. As shown in Fig. 1d, incubation LPS or insulin alone yielded particles ranging from 100 to 1000 nm in diameter, with no discernible variations over the incubation period. By contrast, mixing LPS with insulin led to a gradual time-dependent reduction in the hydrodynamic diameter, implying a direct molecular interplay between LPS and insulin. These results clearly demonstrated that insulin modulates LPS assembly. To interrogate the reciprocal effect of LPS on insulin, we performed enzyme-linked immunosorbent assay (Elisa) to quantify insulin immunoreactivity, revealing that LPS impairs antibody recognition of insulin (Fig. 1e). Therefore, our data establish a direct and bidirectional interaction between insulin and LPS.

To test whether the observed interaction arises from specific molecular recognition and to quantify the binding affinity between insulin and LPS, we performed surface plasmon resonance (SPR) spectroscopy assay using biotinylated LPS immobilized on a streptavidin sensor chip. Kinetic binding parameters were derived using the average molecular weight of LPS (approximately 20 kD), yielding an equilibrium dissociation constant (K_D) of 1.54 μ M (Fig. 1f). Similar affinities were obtained by tagging free insulin on a CM5 sensor chip for LPS binding (Extended Data Table 1). As numerous engineered insulin analogues have been developed to replace native insulin, we systematically assessed their binding profiles to LPS.

The results demonstrated that, with the exception of glargine, whose behavior may be explained by its neutral isoelectric point value, the other three analogues exhibit comparable LPS binding affinities relative to native insulin (Extended Data Fig. 1a).

Next, we evaluated the binding between insulin and LPS in serum. Normal and *E. coli* infected Wistar rats received intraperitoneal glucose injections, then LPS was affinity-purified from serum samples using polymyxin B (PMB)-agarose beads. The presence of insulin in the LPS-enriched fractions was examined by Western blotting and Elisa. The results revealed a significantly greater abundance of insulin co-purified with LPS from septic rats (Fig. 1g,h), indicative of complex formation between insulin and LPS *in vivo*.

Divalent cations exert propound effects on the structural stability of both LPS and insulin^{19,20}. We therefore investigated their influence on the insulin-LPS interaction using a panel of chemical modulators. As shown in Extended Data Table 1, none of the tested cations affect the association between LPS and insulin. By contrast, Triton X-100, a nonionic surfactant, significantly reduced the binding affinity of insulin for LPS, implying that hydrophobic interactions underpin their molecular association.

LPS reduces the physiologic function of insulin

Insulin is essential for maintaining systemic glucose homeostasis. In response to insulin stimulation, both hepatocytes and skeletal muscle cells exhibited marked increases in the absorption of 2-NBDG, a fluorescent glucose analogue²¹. Relative to the control group, LPS had minor influence on 2-NBDG absorbance (Fig. 2a,b). However, exposure to insulin-LPS complex (IL) led to a pronounced reduction in 2-NBDG internalization. Glucose uptake was further approximated via quantification of intracellular glycogen content. Periodic acid-Schiff staining (PAS) (Fig. 2c) and anthrone-based colorimetric quantification revealed that glycogen deposition was significantly diminished in IL-treated groups compared with insulin-stimulated counterparts (Fig. 2d).

The *in vivo* effect of LPS on insulin activity was investigated in male C57BL/6 mice by performing intraperitoneal insulin tolerance tests (ipITTs). No fluctuations in blood glucose levels were observed over the 60-minute LPS administration period (Extended Data Fig. 2a). Time-course profiles of absolute blood glucose levels during ipITTs, along with the corresponding calculation of the area under the curve (AUC), were presented in Fig. 2e. Upon insulin injection, blood glucose levels exhibited a dramatic and rapid drop. Glucose-lowering trends in groups co-administered insulin and LPS were comparable to those in insulin group, albeit attenuated. The hypoglycemic efficacy of insulin was significantly diminished when the insulin: LPS molar ratio lowered to the ratio of 2.5:1. In intraperitoneal glucose tolerance tests (ipGTTs) (Fig. 2f), LPS challenge dose-dependently impaired glucose clearance, resulting in a marked elevation in the area under the ipGTT curve.

Based on the observed LPS-related glucose elimination abnormalities, we proceeded to assess the potential impact of glycogen synthesis in both the liver and skeletal muscle. PAS colorimetric staining of liver tissue slices post-ipGTT analysis showed that LPS reduced insulin mediated glycogen accumulation (Fig. 2g). This result was confirmed by the glycogen content assay results (Fig. 2h). Notably, similar results were observed in skeletal muscles, suggesting a consistent influence of LPS on impairing glycogen synthesis across different tissues. Thus, these findings imply that LPS compromises the physiological activity of insulin.

LPS blocks insulin signal transduction

To comprehend the intricacies of LPS-induced insulin resistance, we assessed the expression and phosphorylation levels of insulin receptor (INSR), protein kinase B (AKT) and glycogen synthase kinase-3 β (GSK-3 β) in HepG2 hepatocytes and C2C12 myotubes (Fig. 3a). In contrast to the control group, insulin strongly triggered the phosphorylation of the INSR β -subunit, AKT (S473) and GSK-3 β within both cell types. While, LPS dose-dependently suppressed the stimulation effect of insulin (Extended Data Fig. 2b). In parallel, LPS inhibited signaling mediated by insulin analogues (Extended Data Fig. 1b).

Insulin stimulation has been shown to promote glucose transporter 4 (Glut4) translocation from the intracellular compartments to the plasma membrane, thereby enhancing glucose absorption. As illustrated in Fig. 3b, neither insulin nor LPS modulated the total cellular abundance of Glut4. Insulin alone stimulated Glut4 traffic to the membrane, accompanied by a concomitant decrease in its cytoplasmic localization. Strikingly, co-treatment with LPS reduced the insulin-evoked translocation of Glut4.

LPS prevents insulin recognition by its receptor

The above results suggested that LPS may interfere with the recognition of insulin by the insulin receptor (INSR). We therefore assessed the binding capacity of insulin to INSR. INSR was immobilized on Elisa plates to directly assess the binding of FITC-insulin to the receptor. Consistent with the underlying hypothesis, the results demonstrated that LPS concentration-dependently abrogated the binding interaction between FITC-insulin and INSR (Fig. 3c). We next examined cellular responses, and in cells treated with FITC-insulin, prominent fluorescence signals were observed, indicative of robust-receptor binding. In contrast, LPS reduced cellular FITC fluorescence intensity. Quenching of membrane-associated fluorescence with trypan blue revealed that insulin internalization was modestly reduced upon co-incubation with LPS (Fig. 3d).

Next, we analyzed insulin binding to the INSR in target tissues using a septic animal model. While bacterial infection induced no measurable shifts in circulating insulin and C-peptide concentrations (Fig. 3e,f), *in vitro* assays revealed that LPS suppresses insulin-degrading enzyme (IDE)-mediated insulin degradation (Fig. 3g). Given that LPS interferes with Elisa-based insulin quantification, these

observations imply that true insulin concentrations in infected mice exceed those measured in healthy controls. Immunofluorescent visualization of membrane-associated insulin in liver and skeletal muscle sections revealed a marked reduction in insulin binding to both tissues in infected rats relative to non-infected controls (Fig. 3h). Collectively, LPS impairs the tissue-targeting efficacy of insulin, rather than affecting insulin secretion per se.

Lipid A moiety of LPS is responsible for insulin inactivation

To dissect the potential components governing LPS binding and its interference with insulin action, we performed a comparative analysis using LPS derived from distinct genera. Despite slight variations, the three LPS variants exerted inhibitory effects on insulin signaling cascades and insulin-mediated glucose uptake (Fig. 4a). Next, we isolated the *O*-antigen fraction from *E. coli* O111:B4 LPS and validated the absence of contaminating lipid A by monitoring toll-like receptor 4 (TLR4) expression (Extended Data Fig. 3a). We observed that the isolated *O*-antigen fraction failed to attenuate insulin activity (Fig. 4b). Kdo2-lipid A (KLA), the bioactive core of LPS, acts as a potent pro-inflammatory stimulant *via* TLR4²². Co-incubation of insulin with KLA similarly resulted in significant impairments to insulin signal transduction and cellular glucose uptake. To dissect whether LPS phosphate moieties contribute to insulin suppression, we employed divalent cations (Ca^{2+} and Mg^{2+}) to sequester phosphate anions. In line with the SPR data, neither cation abrogated LPS-mediated inhibition (Fig. 4c). While LPS derived from *Porphyromonas gingivalis* (LPS-PG) differed substantially from *E. coli*-derived LPS in phosphate moieties and the number, length and distribution of acyl chains, it still exerted weak inhibitory effects (Fig. 4d). In parallel, we further showed that the activity of LPS was competitively mitigated by the LPS antagonist PMB²³ (Fig. 4e). Collectively, these findings confirmed that lipid A mediated the inhibition effect of LPS independent of the *O*-antigen and phosphate moieties, and primarily depends on the acyl chains of lipid A.

Insulin-LPS acts independently of TLRs and precedes pro-inflammatory cytokines releasing Given that LPS can promote insulin resistance through inflammatory signaling, we sought to understanding the implications of LPS-induced inflammation in this interplay. TLR4 is the primary receptor mediating LPS recognition and subsequent inflammatory activation. We first detected the time course of TLR4 activation by LPS in HepG2 cells. As shown in Extended Data Fig. 3b, TLR4 expression was time-dependent. It was undetectable until 1.5 h after LPS induction, with this temporal onset lagging behind of the activation of insulin signaling cascades. To further illustrate the association of TLR4, we used small interfering RNA (siRNA) to silence TLR4 expression in HepG2 cells (Extended Data Fig. 3c). TLR4 silencing impaired cell survival (Extended Data Fig. 3d) and attenuated cellular responsiveness to insulin stimulation (Fig. 5a). Notably, in TLR4-silenced cells, LPS exhibited an insulin signaling inhibition profile comparable to that in wild-type cells. Further support came from primary hepatocytes isolated from TLR4 knockout mice (Extended Data Fig. 3e), which confirmed that LPS interfered with insulin physiological function and signal transduction independently of TLR4 (Fig. 5b,c). TAK-242 (resatorvid), a TLR4 antagonist that specifically binds to the intracellular domain of TLR4 and inhibits LPS-induced

signaling²⁴, exerted a dose-dependent inhibition of LPS-induced TLR4 expression (Extended Data Fig. 3f). The addition of TAK-242 had no appreciable effect on the functionality of the insulin-LPS complex (Fig. 5d).

In vivo experiments were conducted to examine whether LPS can act independently of TLR4 in regulating insulin sensitivity. Before functional assessments, baseline blood glucose and insulin concentration were compared between *Tlr4*^{-/-} and wild-type mice. After a 6 h fast, no significant differences were observed between the two groups (Extended Data Fig. 3g,h). At an LPS dosage that did not modify blood glucose concentrations in *Tlr4*^{-/-} mice (Extended Data Fig. 3i), results from both the ipGTT and ipITT assays demonstrated that LPS-induced insulin resistance was unaffected by the loss of TLR4 (Fig. 5e,f). Consequently, hepatic glycogen accumulation in *Tlr4*^{-/-} mice was decreased following LPS administration (Extended Data Fig. 4a,b). This TLR4-independent effect was also recapitulated in *Tlr2*^{-/-} mice (Extended Data Fig. 4c,d). Therefore, neither TLR4 nor TLR2 contributes to the LPS-interaction mediated attenuation of insulin activity.

To exclude the possibility that LPS elicits inflammatory factors production via alternative pathways, serum levels of inflammatory cytokines during the ipGTT were quantified by Elisa. The analyses revealed that TNF- α , IL-6, and IL-1 β levels exhibited no significant alterations at 30 minutes post LPS administration (Fig. 5g). At 60 minutes point, the levels of TNF- α , and IL-6 were significantly elevated, whereas IL-1 β remained unaltered. No significant differences were detected between the LPS and IL groups, suggesting that the modulation of insulin on LPS biological behavior was not significant during the time interval. We further examined the effects of the three pro-inflammatory cytokines on insulin signaling. Notably, 30 min stimulation with each cytokines at 400 pg ml⁻¹ failed to impair this cascade (Extended Data Fig. 5a), whereas elevated concentrations exerted suppressive effects (data not shown). Integrating the ipGTT glycemic profiles with the kinetics of inflammatory cytokines release, it is evident that the binding to LPS-induced insulin resistance preceded the production of these proinflammatory cytokines. To confirm this observation, we performed RNA Sequencing (RNA-Seq) analysis (Extended Data Fig. 5b). Although transcriptomic profiling seemed that the complex treatment activated the pro-inflammatory and pro-proliferative SENP3-EIF4A1-CCN1-AP1 positive-feedback axis while repressing CMC2 and DEPP1 transcription, subsequent qPCR verification demonstrated that such upregulation failed to achieve statistical significance (Extended Data Fig. 5c). Thus, the gene transcription assay further confirmed that LPS binding to insulin induced insulin resistance prior to the release of inflammatory cytokines.

High-density lipoprotein (HDL) involves in the clearance of insulin-LPS complex

To investigate the metabolic pathway of the insulin-LPS complex *in vivo*, we focused our attention on lipoproteins. Quantification of LPS and insulin within lipoprotein fractions isolated via sodium bromide density gradient ultracentrifugation revealed that their abundances increased upon combined exposure

conditions (Fig. 6a). High-density lipoproteins (HDL) were reported to neutralize over 80% circulating LPS by directly binding to the lipid A component of LPS²⁵. On this basis, we investigated the correlation between HDL and the insulin-LPS complex. Apolipoprotein A1 (Apoa1) serves as the primary structural and functional protein of HDL and immunoprecipitation analysis verified that Apoa1 forms complex with insulin in a LPS-dependent manner (Fig. 6b). To dissect this process, we purified endogenous HDL via density-gradient ultracentrifugation (Extended data Fig. 6a,b), and deployed Förster resonance energy transfer (FRET) to quantify cellular endocytosis of the insulin-LPS complex, using FITC-insulin as the fluorophore donor and CF594-LPS as the acceptor (Extended data Fig. 6c and Fig. 6c). It showed that co-exposure to HDL induced a strong, time-dependent FRET signal with dual probes, whereas negligible fluorescence was observed in single-probe groups. Furthermore, individual insulin or HDL exposure promoted cellular LPS uptake, and combined treatment generated synergistic potentiation of LPS internalization. Though free LPS suppressed insulin cellular entry, co-incubation with HDL completely abrogated this inhibition and boosted insulin endocytosis (Fig. 6d). Together, these data suggest HDL facilitates the uptake of LPS-insulin complexes, which was corroborated by confocal imaging showing prominent intracellular co-localization of LPS, insulin, and Apoa1 in HDL-containing cultures (Fig. 6e and Extended Data Fig. 6d). Scavenger receptor class B type I (SR-BI) is a primary receptor of HDL²⁶. The reliance of insulin clearance to SR-BI was evaluated with BLT-1, a specific SR-BI inhibitor (Extended Data Fig. 7a,b). The results proved that BLT-1 markedly suppressed cellular uptake of insulin (Fig. 6f).

To observe the *in vivo* interaction between LPS and insulin, we constructed a rat model of dextran sulfate sodium (DSS)-induced intestinal barrier dysfunction (Extended Data Fig. 8). Heat inactivated *E. coli* strain and glucose were co-administered by oral gavage to simulate dietary intake. Liver tissue was harvested and analyzed by immunofluorescence staining. In line with above results, immunofluorescence confirmed robust insulin-LPS colocalization in the liver slices (Fig. 6g).

Discussion

It has been reported that LPS was found in 100% of the pancreatic specimens from patients of T2DM, in contrast to only 25% from those without T2DM²⁷. For decades, the prevailing paradigm has emphasized the central contribution of proinflammatory cytokines (TNF- α , IL-6, and IL-1 β) to LPS-induced insulin resistance. However, in this work we challenged this canonical framework by demonstrating that LPS induces insulin resistance through a distinct, inflammation-independent mechanism. Specifically, LPS binds with insulin, thereby preventing the receptor from perceiving this hormone. This binding disrupts the normal downstream signaling cascades, including the phosphorylation of INSR, AKT, GSK-3 β , as well as the translocation of Glut4 to the membrane. Ultimately, it impairs the uptake and utilization of glucose by target cells and tissues, leading to insulin resistance.

Our results suggest that the conserved moiety, lipid A, of LPS, serves as the key factor in mediating the interplay between LPS and insulin. This structural specificity provides a molecular basis for the conserved metabolic disturbances induced by diverse intestinal gram-negative bacteria, and suggests

that targeting intestinal LPS translocation and lipid A-insulin interaction may serve as a prevention strategy to alleviate endotoxin-associated metabolic dysfunction.

The result that LPS-insulin mediated insulin desensitization occurs at an early time window post LPS exposure, distinguishes the direct metabolic toxicity of LPS from its secondary inflammatory injury. This provides a novel mechanistic insight into the clinical paradox that metabolic dysfunction and hyperglycemia can arise under negligible or low-grade inflammatory conditions^{28–30}, alongside limited efficacy of anti-inflammatory interventions in a subset of obese and diabetic individuals^{31,32}.

Another conceptual advance of this work is the identification of the HDL-SR-B1 axis as a resolution pathway for LPS-induced insulin dysfunction. This mechanism reveals a novel role for HDL in modulating systemic insulin turnover under endotoxin stress. Unlike inflammatory signaling, which acts primarily to inhibit intracellular insulin signal transduction, the HDL-mediated clearance pathway represents a previously unrecognized extracellular regulatory layer that controls blood insulin levels, providing a distinct mechanistic basis for acute-onset insulin resistance triggered by LPS. Impairment of this clearance axis—for instance, under conditions of hypo-HDL cholesterolemia or SR-B1 dysfunction—may therefore exacerbate hyperinsulinemia, a common feature observed in the early stage of T2DM^{33–36}.

Integrating our observations, we support a two-phase model of LPS-induced metabolic dysfunction. The early, acute phase is driven by lipid A-dependent modulation of HDL-mediated insulin clearance, triggering insulin depletion and insulin resistance in the absence of inflammation. The later chronic phase is superimposed by canonical inflammatory cytokine release, which further exacerbates insulin signaling defects. This model revises the conventional inflammation-centric framework of endotoxin-induced metabolic disorder, highlighting lipid A as a upstream regulator that initiates metabolic perturbation via a unique non-inflammatory axis.

Methods

Animals Male C57BL/6J mice (8–12 weeks old) and male Wistar rats (6–8 weeks old) were purchased from the Qingdao Qinda Biology Science and Technology Co., Ltd. (Rodent license No. SCXK 2019-0003) and kept in isolated cages in a barrier animal facility in temperature-controlled (20–22 °C) room. Male *Tlr4*^{-/-} and *Tlr2*^{-/-} C57BL/6J mice were kindly provided by Professor Chen-Yang, Zhao. Cages were kept in a standard light/dark cycle with ad libitum access to food and water. Blood glucose levels were determined with ACCU-CHEK Performa blood glucose meter (Roche Diagnostics, Switzerland). The experiment was conducted as a completely randomized design, and the analyses were performed in a blinded manner. Procedures were approved by the Ethical Committee (rodent license number OUC-SMP-2023-0403).

Cell culture Human hepatocellular carcinoma HepG2 cells, mouse myoblast C2C12 cells were purchased from the Chinese Academy of Sciences Stem Cell Bank (Shanghai, China). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum.

iBMDM cells were kindly provided by professor Feng Shao (National Institute of Biological Sciences, Beijing).

For differentiation into myotubes, C2C12 cells were incubated with 2% (v/v) horse serum in the DMEM for 5–7 days until 80% confluence³⁷. Then, the differentiated C2C12 cells are used in subsequent experiments.

Isolation and culture of primary hepatocytes Hepatocytes from wild or *Tlr4*^{-/-} C57BL/6J mice were isolated by collagenase perfusion method as described previously³⁸ and cultured for 2 days in the DMEM.

Generation of genetically engineered cells To knock down the TLR4 gene expression, HepG2 cells were transfected with TLR4 small interfering RNAs (siRNAs) or the scrambled control for 48 h. The siRNAs were synthesized by TSINGKE Biological Technology (China) (Extended Data, Table 5). The expression of TLR4 was validated by Western Blot analysis. A cell counting kit-8 (CCK-8) was used to test the cell viability of TLR4 knocking down cells, according to the manufacturer's protocol.

Surface plasmon resonance (SPR) assay The binding ability of insulin to Biotin-EB LPS was analyzed using a BIAcore T200 instrument (GE Healthcare) with streptavidin (SA) sensor chips. For LPS binding to insulin, insulin was immobilized on a CM5 sensor chip surface. Buffer PBS (pH7.5) was used as the running buffer and sample dilution buffer. Serially diluted LPS or insulin sample was injected into the analysis channel and passed over the sensor chip at a flow rate of 30 $\mu\text{l min}^{-1}$. For all samples, response curves were also recorded on control surfaces. Results were calculated after subtraction of the control values using the BI Data Analysis software. The affinity constant (K_D) was derived from the interaction between insulin and LPS. The molar concentration of LPS was calculated with an average molecular weight of 20 kDa, calculated by gel permeation chromatography.

Pull-down of serum insulin In the control group, Wistar rats (8 weeks old, n = 10 per group) were caudal vein injected (i.v.) PBS and in the septic group rats were injected by *E. coli* ATCC 25922 (5×10^8 cfu 150 g^{-1}). After starving for 6 h, glucose (2 g kg^{-1}) was intraperitoneal administered (i.p.). Fifteen minutes later, blood samples were collected from heart using a negative pressure tube. Serum was prepared by centrifugation at 2000 rpm for 10 min. Then, phenylmethylsulphonyl fluoride (PMSF) and protease inhibitor cocktail were added to the serum. Serum samples were then incubated with PMB-agarose beads at room temperature for 2 h. After being washed 3 times with PBS, the beads were eluted with 0.1 M glycine-HCl (pH 2.5). Insulin in the collected eluent was analyzed using Western Blot analysis and Elisa analysis.

Enzyme-linked immunosorbent assay Elisa was used to measure the levels of serum cytokines. Briefly, serum was obtained through centrifuging the blood samples from the tail vein. Then, Elisa kits were used to detect insulin, C-peptide or proinflammatory cytokine levels following the instructions.

Dynamic light scattering (DLS) assay DLS was recorded using a Zetasizer Nano (Malvern Instruments, Worcestershire, UK). The radii of LPS, insulin, and insulin-LPS complex were determined after incubation at 37°C for various time intervals¹⁵. Data evaluation was performed by GraphPad Prism 8.0 software.

Zinc salt staining assay LPS and/or insulin diluted in PBS was dripped into the sample hole of 0.8% (w/v) agarose gel and operated at 80 V in the TAE buffer. After running, gel was stained with imidazole as previously described¹⁷. Briefly, the gel was washed with ddH₂O for 3 times, and then oscillated for 20 min in 0.2 M imidazole. After washing with ddH₂O, the gel was placed in the dark and shaken 30 s with 0.2 M zinc sulfate solution. Finally, the gel was washed with ddH₂O to stop staining. For double staining experiments, after scanning the gel image of the reverse staining method, the gel was stained with 0.25% (w/v) Coomassie brilliant blue solution.

Insulin and LPS complex formation For insulin and LPS complex formation, the two molecules were incubated at room temperature for 10 min.

Flow cytometry assay Flow cytometry was used to detect the binding ability of insulin and its receptor. After culture, cells were collected with EDTA-free trypsin (0.25%) and washed twice with ice-cold PBS. Then, FITC-insulin or FITC-insulin premixed with LPS was incubated with cells for 0.5 h at 37°C. Following 3 times washing, the fluorescence of cells was detected by a flow cytometer (Novocyte, Agilent, USA) with green fluorescent light at a wavelength of 488 nm. To quench the membrane fluorescence, trypan blue was supplied at final concentration of 0.2% before flow cytometry analysis³⁹.

Glucose uptake assay HepG2 cells or C2C12 myotube cells (5×10^5 cells ml⁻¹) were starved for 6 h in the serum-free low glucose DMEM medium. Then cells were treated with the indicated reagents and incubated with 2-NBDG (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose) (100 µg ml⁻¹) for 30 min at 37°C⁴⁰. The reaction was stopped by removing the incubation medium and washing with pre-chilled PBS for three times. The Fluorescence intensity of 2-NBDG was measured at 466/550 nm by flow cytometry (Novocyte, Agilent, USA) and analyzed with NovoExpress software.

Glycogen content assay For glycogen analysis in cells, both HepG2 and differentiated C2C12 cells were starved for 6 h before the test. Then, cells were treated with insulin (100 nM), LPS (100 nM), or the complex mixture for 2 h. To detect the glycogen content of liver tissue, after overnight fasting, mice were i.p.-injected with glucose (2 g kg⁻¹) and caudal vein injected with LPS (20 µg kg⁻¹). One and a half hours later, mice were sacrificed and liver tissues were fixed in 4% (w/v) paraformaldehyde. Glycogen content was quantitatively detected by glycogen content detection kit⁴¹. The relative levels of glycogen were normalized to the protein content. For qualitative detection, cells or tissue paraffin sections were stained with periodic acid-Schiff staining reagent for 10 min⁴² and images were captured using microscopy (IX-71, Olympus, Japan).

Intraperitoneal glucose tolerance test (ipGTT) assay After 6 h fasting (deprivation of food with ad libitum access to water), male C57BL/6J mice were bled from the tail vein for a baseline measurement

using a glucometer (Accu-Chek, Roche, Brazil). GTT was conducted by i.p. delivering LPS (2 or 20 $\mu\text{g kg}^{-1}$) or PBS, subsequently glucose (2 g kg^{-1})⁴³. Blood glucose levels of mice were measured at 5, 10, 15, 30, and 60 min. Time versus glucose level was plotted for each animal to calculate the area under the curve (AUC) using GraphPad Prism 8.0 software.

Intraperitoneal insulin tolerance test (ipITT) assay The dynamic characteristics of blood glucose after insulin administration were assessed by intraperitoneal injection of insulin (60 $\mu\text{g kg}^{-1}$) or insulin: LPS in molar ratio of 2.5:1 or 5:1. Blood glucose levels were determined at 5, 15, 30, and 60 min after injection. After the experiment, mice were supplied with glucose 2 g kg^{-1} and then ad libitum access to food and water.

Insulin degradation assay Insulin (1 μM), either alone or mixed with LPS, was incubated with insulin-degrading enzyme (100 nM) at 37°C for degradation reactions.

Detection of insulin binding to the target tissue Rats were i.p.-infected with *E. coli* 25922 (5×10^8 cfu 150 g^{-1}) and then starved for 6 h. After glucose administration for 15 min, rats were sacrificed. Fresh liver tissues and gastrocnemius muscles were taken and fixed in 4% Paraformaldehyde for 20 h at 4°C, then dehydrated with 20% sucrose for 20 h. The frozen sections of 5 μm were then prepared using a Leica cryostat (CM1850, Germany) and stained with anti-insulin antibody. To avoid staining of intracellular insulin, the staining process was conducted without permeation agent. After antibodies incubation, the nuclei of cells were stained with 4'-diamidino-2-phenylindole (DAPI). Cells were mounted in anti-fluorescence quenching sealing solution and observed with a confocal microscope (Leica TCS SP8 STED, Germany).

RNA sequencing analysis HepG2 cells were treated with insulin, LPS or IL, each at a concentration of 100 nM, for a duration of 30 min. RNA-seq analysis was performed by Beijing Tsingke Biotech Co., Ltd..

Lipoprotein preparation Lipoproteins were isolated from rat serum by discontinuous sodium bromide (NaBr) density-gradient ultracentrifugation. Briefly, serum was adjusted to a density of 1.063 g ml^{-1} prior to centrifugation, after which the buoyant fraction containing VLDL, IDL, and LDL was harvested. The HDL-rich infranatant retained following this initial spin was subsequently brought to 1.21 g ml^{-1} and subjected to a second ultracentrifugation step, enabling intact HDL particles to float to the meniscus for collection^{44,45}. The prepared lipoproteins were dialyzed against PBS at 4°C.

Förster resonance energy transfer analysis Prior to cell incubation, FITC-insulin, CF594-LPS, and HDL were co-incubated at 37°C for 3 h. After incubating cells with the mixture for designated time intervals, culture plates were washed three times with PBS. FRET fluorescence signals were then recorded using a microplate reader at an excitation wavelength of 488 nm and emission wavelength of 625 nm.

Immunofluorescence detection After incubation with insulin, LPS and HDL for 30 min, cells were fixed and immunolabeled with primary antibodies targeting insulin, LPS, and ApoA1, respectively. Cells were then blocked with 5% BSA at room temperature to block non-specific antibody binding, followed by

incubation with corresponding fluorescent secondary antibodies for 1 h. Cell nuclei were counterstained with DAPI, and coverslips were mounted using anti-fade mounting medium. Laser scanning confocal microscopy was utilized to capture fluorescence images for analysis of intracellular co-localization among insulin, LPS, and ApoA1.

Leaky gut model Acute colitis was induced by oral gavage of 5% DSS for 7 days⁴⁶. *E. coli* 25922 was incubated at 55°C for 30 min to ablate viable bacterial cells. Following heat inactivation, the bacterial suspension was mixed with glucose and delivered to rats via intragastric gavage. Then, the liver of rat was taken out and detected by immunofluorescence after 30 min of treatment.

Statistical analysis All experiments were performed in triplicate. Data were expressed as mean values $\pm$ standard derivation (SD). The statistical and plotting software package GraphPad Prism (version 8.0) was used to perform Mann-Whitney test, unpaired two-tailed Student's t-test, one-way ANOVA, or two-way ANOVA as described in the figures. P values of < 0.05 were considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: not significant.

Declarations

Acknowledgements and funding sources

We thank Professor Chen-Yang, Zhao Ocean University of China for providing the *Tlr4*^{-/-} and *Tlr2*^{-/-} C57BL/6J mice. We also thank professor Feng Shao National Institute of Biological Sciences for providing the iBMDM cells. This work is supported by Qingdao Marine Science and Technology Center (No.2022QNLM030003-2).

Competing interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Ethics approval and consent to participate

All animal experimental protocols of the study were conducted in accordance with the national guidelines for the use of animals in scientific research "Regulations for the Administration of Affairs Concerning Experimental Animals" and were approved by the license of the Care and Use of Laboratory Animals Monitoring Committee of Ocean University of China (rodent license number OUC-SMP-2023-0403).

Contributions

X.L. and W.Y. conceived and designed the experiments, and wrote the manuscript with input from all authors. Z.L., Q.M., L.L., and J.Z. performed the experiments and analyzed the data. F.H. and Q.G. offered valuable insights. W.Y. provided the financial support.

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Figures

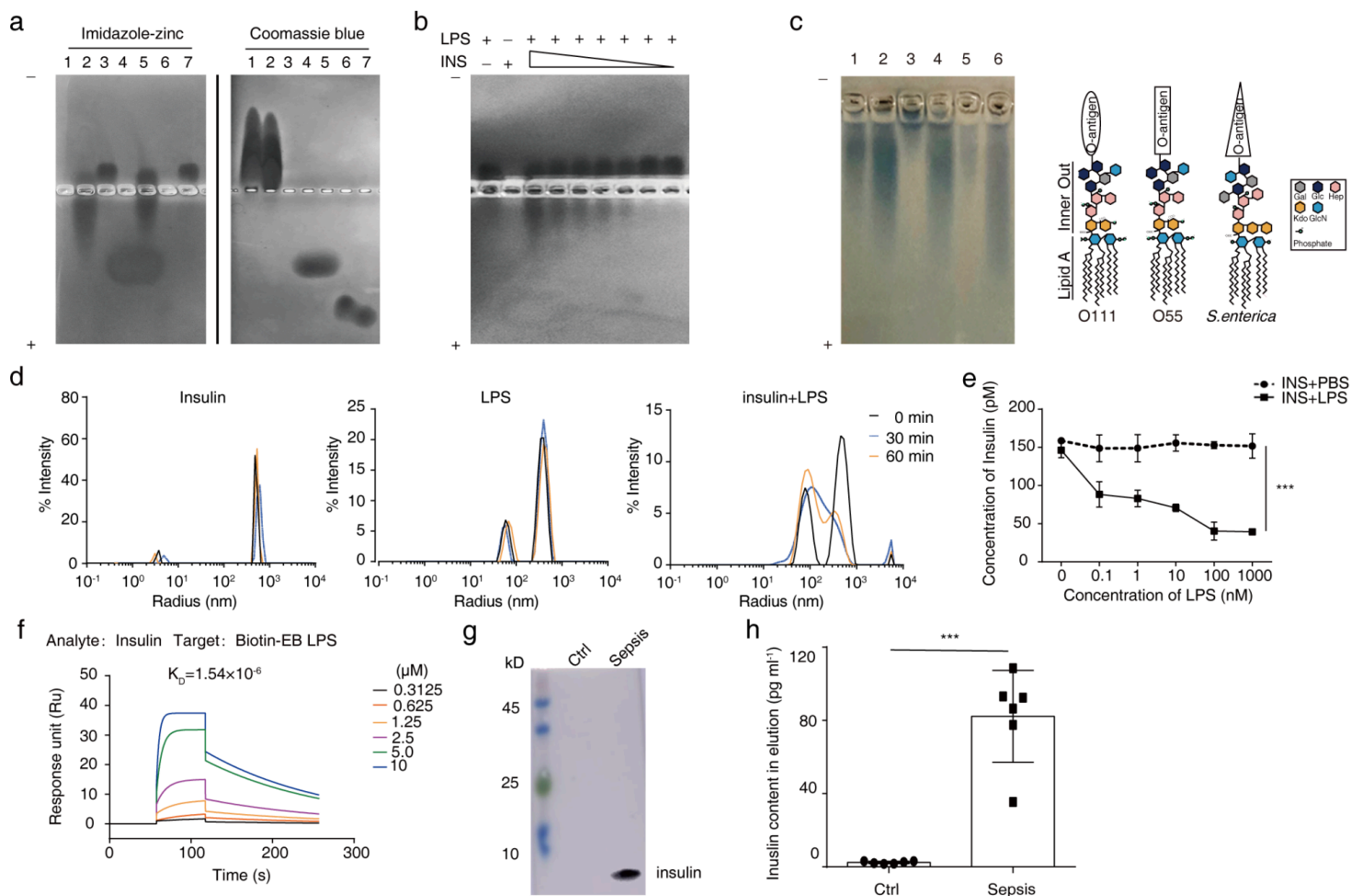


Figure 1

Insulin binds to LPS. (a) Analysis the interaction between insulin and LPS from *E. coli* O111:B4 using native agarose gel electrophoresis. LPS was stained by zinc salt (left) and protein was stained by Coomassie blue (right). Lane 1-7 represents lysozyme (LZM), LZM-LPS mixture, LPS, insulin, insulin-LPS mixture, BSA, and BSA-LPS mixture, respectively. All the mixtures were incubated at 37 °C for 60 min before analysis. (b) The effects of different doses of insulin (2.5, 1.3, 0.6, 0.3, 0.2, 0.1, or 0.05 $\mu g \mu l^{-1}$) on LPS (2.5 $\mu g \mu l^{-1}$) migration. (c) The migration of three types LPS after incubating with insulin. Lane 1-6 represents LPS (*E. coli* O55:B5), insulin-LPS O55:B5, LPS (*E. coli* O111:B4), insulin-LPS O111:B4, LPS (*Salmonella enteritidis*), and insulin-LPS *Salmonella enteritidis*, respectively. (d) Dynamic light scattering (DLS) analysis of the diameter of insulin, LPS, and their mixture after incubating at 37 °C for different duration. (e) Elisa analysis of insulin with different doses of LPS from *E. coli* O111:B4. $n = 3$. Statistical analysis was performed with two-way ANOVA. Data are represented as mean $\pm$ s.d.. (f) Affinity detection insulin binding to LPS using surface plasmon resonance (SPR). Biotin-LPS was immobilized on streptavidin chips. Serially diluted insulin was injected to the analysis channel. (g,h) Detection of insulin pulled down from the serum of Wistar rats. Rats in the sepsis group ($n=10$ per group) were infected with *E. coli* ATCC25922. Glucose (2 g kg^{-1}) was administrated 6 h post-infection to induce insulin release. Serum was collected 15 min later and applied to PMB-agarose beads for pulling down of LPS. Insulin in

pulling down LPS was analyzed by Western Blot (g), and Elisa quantification (h). Data from three biological replicates are presented as mean $\pm$ s.d.. Statistical significance was determined by Mann-Whitney test.

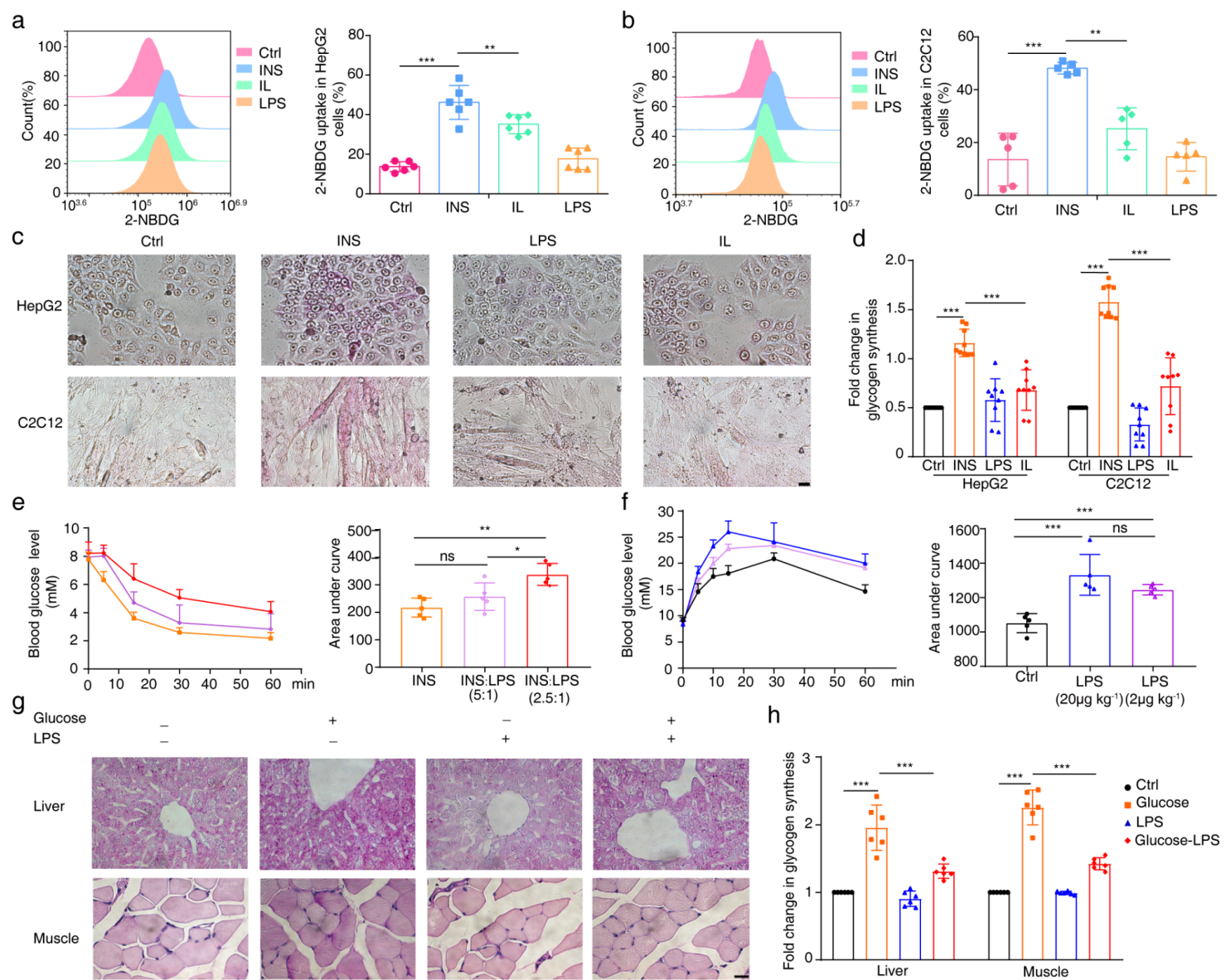


Figure 2

LPS reduces the physiologic function of insulin. (a,b) Glucose uptake of HepG2 cells (a), and C2C12 cells (b) was quantified with 2-NBDG using flow cytometry. Cells were incubated with insulin (INS) (100 nM), LPS (100 nM), or their complex mixture (IL) for 30 min, n=6. (c) Periodic acid Schiff staining of glycogen in HepG2 and C2C12 cells, (Scale bar, 50 μ m). (d) Anthrone quantification of glycogen, n = 9. (e) The hypoglycemic activity of insulin was monitored in wild type C57BL/6J mice (n=5 per group) using the intraperitoneal insulin tolerance test (ipITT). Six hours post-fasting, insulin or insulin: LPS (molar ratio of 2.5:1 or 5:1) was injected intraperitoneally. Then, plasma glucose levels were measured at 0, 5, 15, 30, and 60 min following the challenge, n = 5. (f) The ipGTT analysis was performed by injecting glucose (2 g kg^{-1} , intraperitoneally) with or without LPS (2 μ g kg^{-1} or 20 μ g kg^{-1} , intravenously), n = 5. (g) Glycogen

accumulation in the liver and gastrocnemius muscle during the ipGTTs (Scale bar, 20 μ m). (h) Quantitative analysis of glycogen in the tissues, n = 6. Statistical analysis was performed by one-way ANOVA. Data are presented as mean $\pm$ s.d.

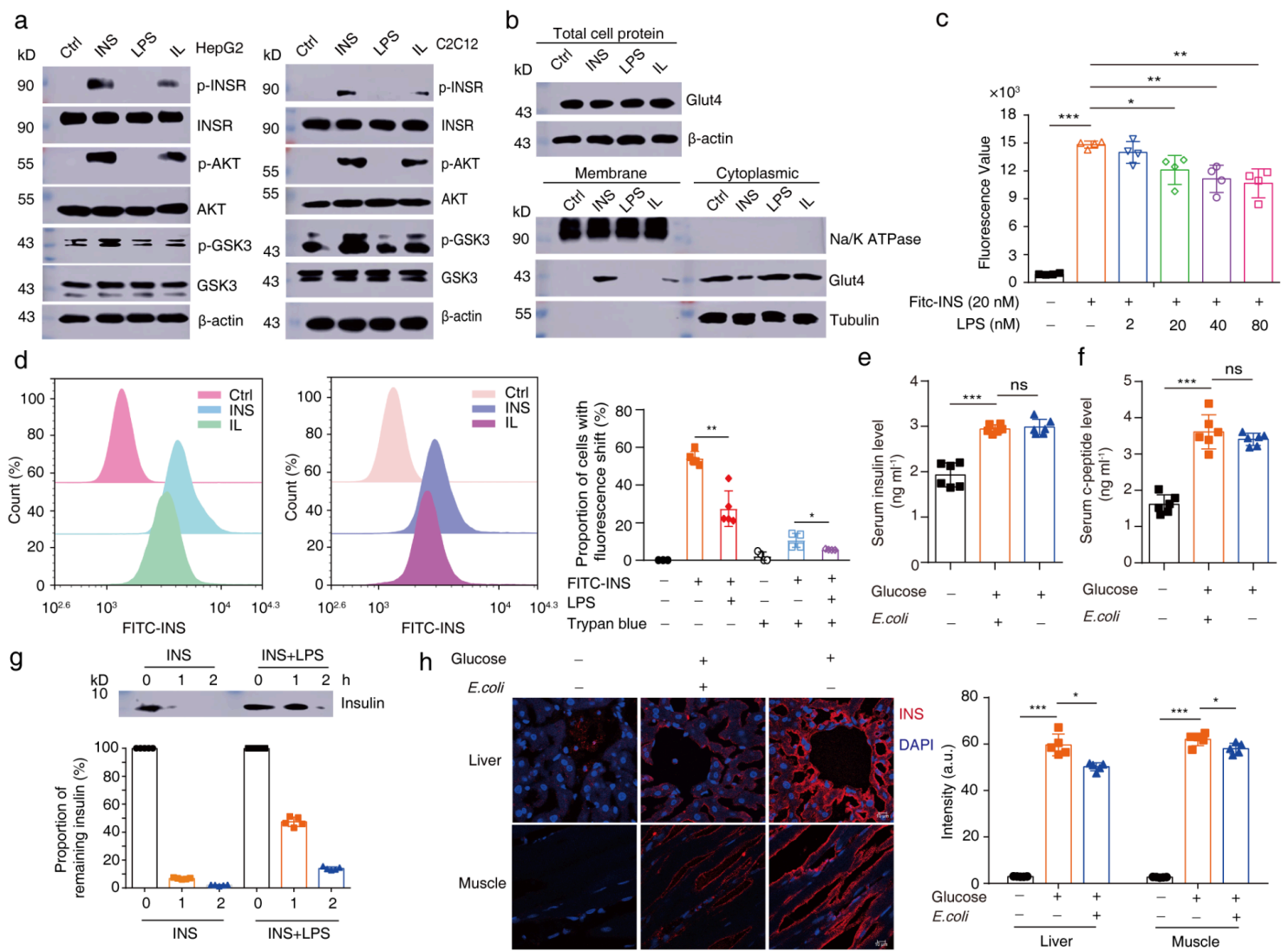


Figure 3

LPS blocks insulin signal transduction by preventing the receptor recognition of insulin. (a) Effects of LPS on insulin signaling in HepG2 and C2C12 cells were detected 30 min post-challenge. The concentration of insulin (INS), LPS, and IL was 100 nM, respectively. (b) Effects of LPS on the expression and distribution of Glut4 in C2C12 myotubes. (c) *In vitro* binding of insulin with insulin receptor. INSR was fixed in a black 96-well plate, and FITC-INS and LPS with different concentrations were incubated for 30 min at 37 °C. After washing with PBS, the fluorescence level was detected by enzyme-labeled instrument. Statistical significance was determined with one-way ANOVA. (d) Flow cytometric analysis of FITC-insulin binding to HepG2 cells. Cells were incubated with FITC-insulin (1 μ M) with or without LPS (1 μ M) at 37°C for 0.5 h. Trypan blue at a final concentration of 0.2% was used for membrane fluorescence quenching. Statistical significance was determined with Mann-Whitney test, n = 5. (e,f)

Serum levels of insulin (e) and C-peptide (f) in rats. Male Wistar rats were infected with *E. coli* 25922 to generate septic models, n = 6 per group. Data are presented as mean $\pm$ s.d. (g) *In vitro* assessment of LPS effects on IDE-mediated insulin degradation. Insulin (1 μ M), either alone or mixed with LPS, was incubated with IDE (100 nM) at 37°C for degradation reactions. (h) Immunofluorescence detection of insulin binding to the liver and skeletal muscle slices. (Scale bar, 10 μ m). The average fluorescence values of different samples were detected by image J. Statistical significance was determined by one-way ANOVA.

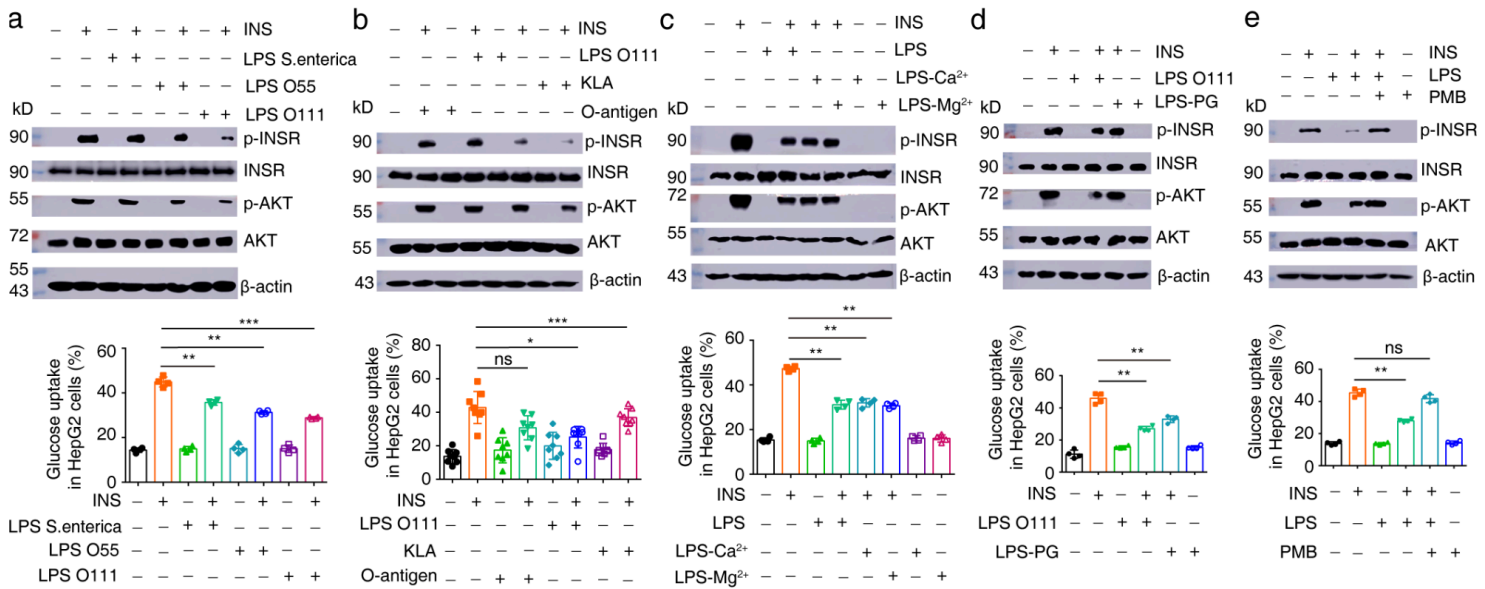


Figure 4

Lipid A moiety of LPS is responsible for insulin inactivation. (a) Effect of different types of LPS on insulin signaling (up) and insulin mediated glucose uptake (bottom). HepG2 cells were challenged with insulin premixed with LPS (2 mg l⁻¹) from *E. coli* O111:B4, *E. coli* O55:B5, or *Salmonella enteritidis*. (b) Effects of O-antigen and Kdo2-lipid A (KLA) on insulin. (c) Effects of divalent cations on LPS interference of insulin. (d) Effects of LPS-PG on insulin. (e) Influence of polymyxin B (PMB) on the inhibition effect of LPS. Data are presented as mean $\pm$ s.d.. Statistical analysis was performed by one-way ANOVA.

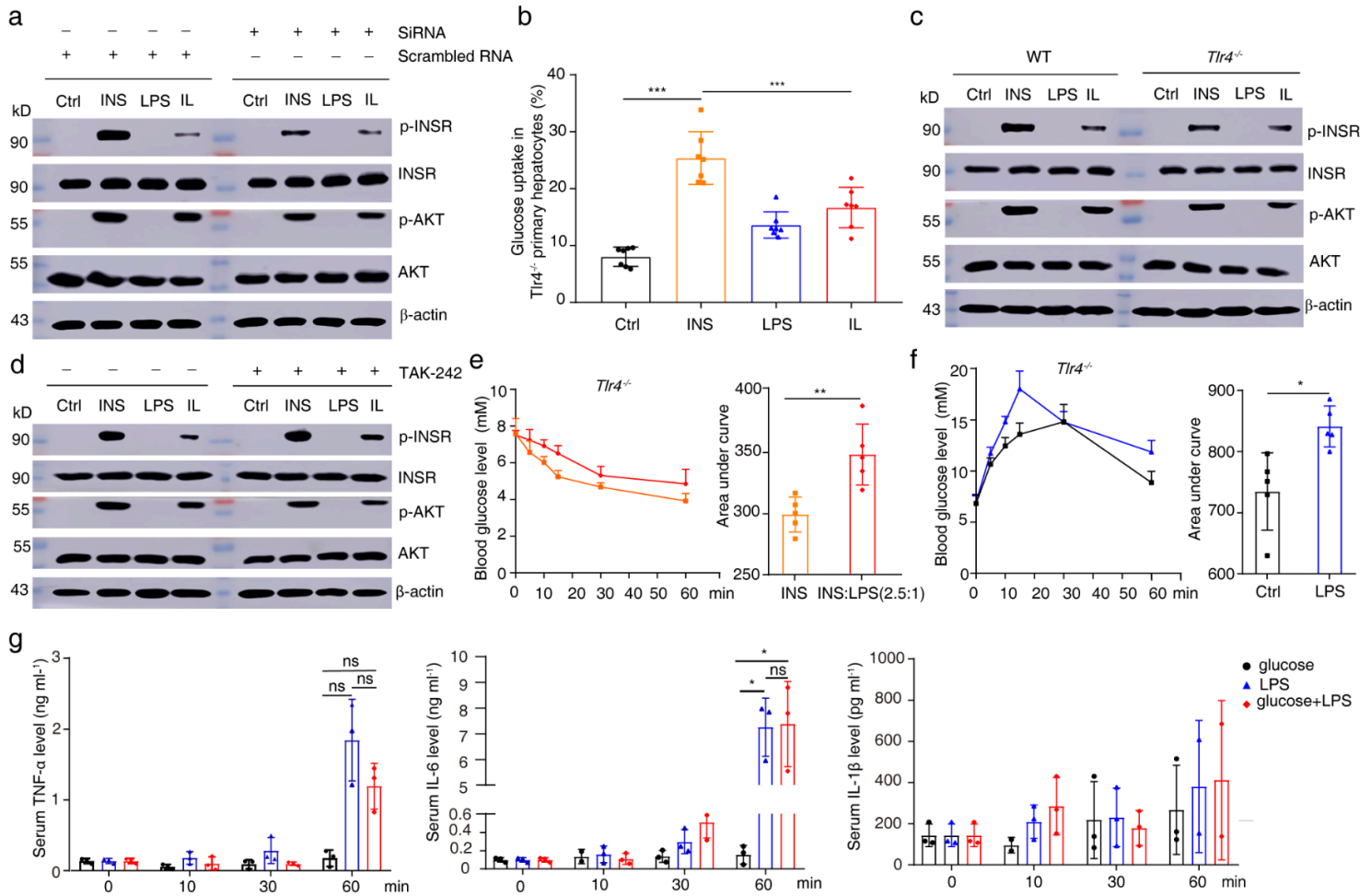


Figure 5

Insulin-LPS complex acts in a TLR4-independent manner. (a) Insulin signaling in TLR4 silenced HepG2 cells. (b,c) Glucose uptake (b), and insulin signaling (c) in primary hepatocytes from $Tlr4^{-/-}$ C57BL/6J mice, $n = 7$. (d) The effects of TAK-242 on LPS-induced insulin resistance in HepG2 cells. Cells were pretreated with TAK-242 (120 ng ml^{-1}) for 3 h before stimulation. (e,f) Effects of LPS on insulin activity in $Tlr4$ knockout mice. ipITT (e), and ipGTT (f) were performed in $Tlr4^{-/-}$ C57BL/6J mice (8-week-old males, $n = 5$ per group). The molar ratio of insulin:LPS in ipITT was 2.5:1. The dose of LPS for ipGTT was $20 \mu\text{g kg}^{-1}$. Data are representative of three independent experiments, $n = 5$. (g) Inflammatory cytokines releasing during ipGTT, $n = 3$. Statistical significance for glucose uptake and AUC was determined by One-way ANOVA and unpaired two-tailed Student's t-test, respectively.

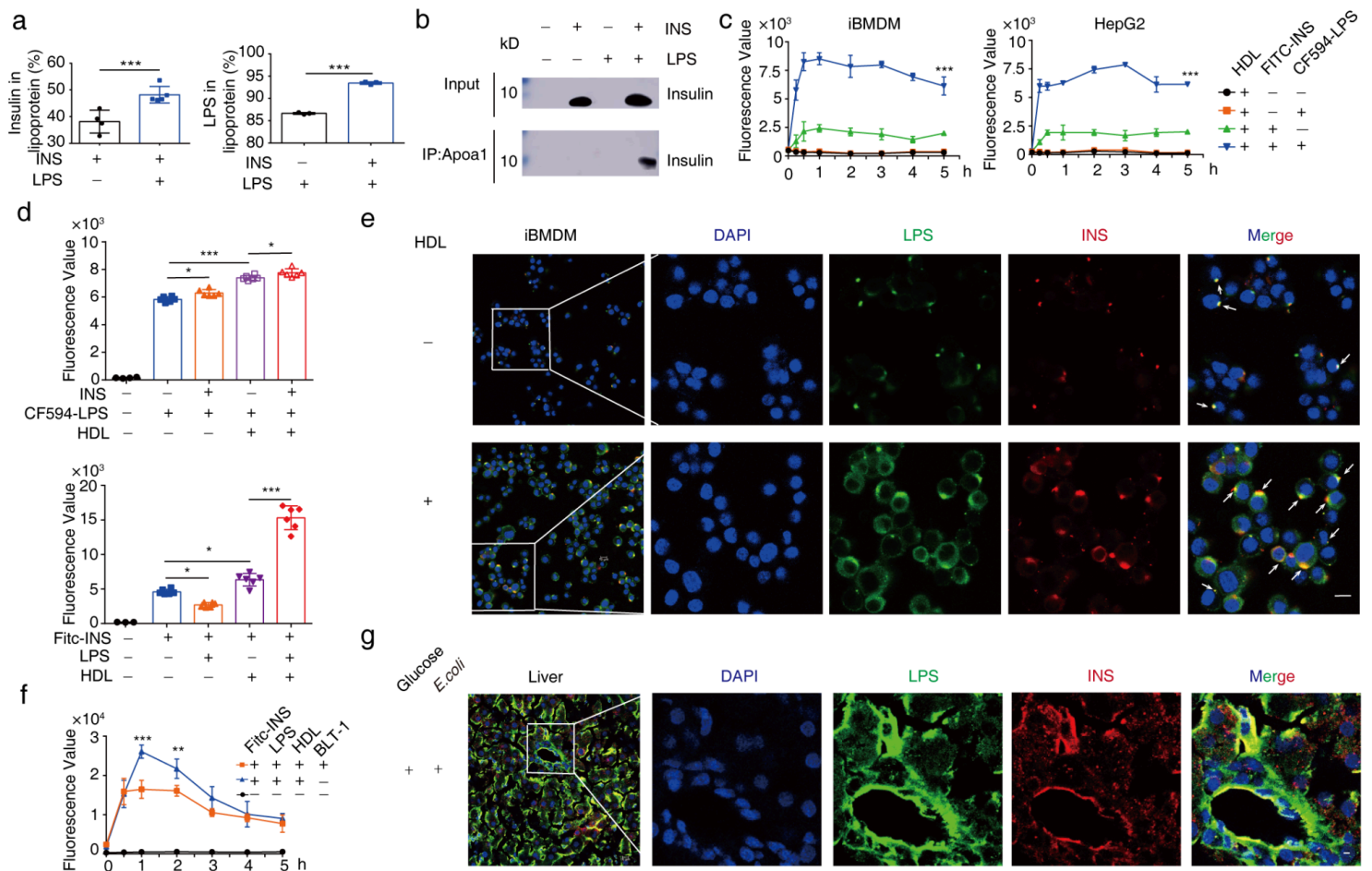


Figure 6

Insulin-LPS complex was sequestered by HDL. (a) Detection of insulin and LPS in ultracentrifugation prepared lipoproteins. Insulin or LPS was mixed with rat serum, followed by lipoprotein separation using sodium bromide density gradient ultracentrifugation. The levels of insulin or LPS were detected by Elisa or LAL, respectively. (b) Immunoprecipitation of insulin with anti-Apoa1 antibody. (c) Detection of cells uptake of insulin-LPS complex using FRET. Fluorescence intensity was measured at excitation 488 nm and emission 625 nm at the indicated time intervals. (d) Quantification of endocytosis of LPS or insulin after incubation with the indicated reagent for 30 min. (e) Immunofluorescence detection of insulin and LPS. Scale bar: 10 μ m. (f) Quantification analysis of SR-B1 inhibitor BLT-1 on cells internalization of insulin. (g) Insulin-LPS colocalization in liver slices of DSS-induced acute intestinal leakage rats. Rats received daily 5% (v/v) DSS oral gavage for 7 days to establish the model, followed by intragastric delivery of glucose and heat inactivated *E. coli* mixture. Livers were harvested 30 min after treatment. Scale bar: 10 μ m. Statistical significance was determined by One-way ANOVA and unpaired two-tailed Students't-test, respectively.

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