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LPS-induced biomarkers in mice: A potential model for identifying insulin sensitizers

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Abstract

The contribution of nutrient overload and associated inflammation to insulin resistance has highlighted several therapeutic targets including c-Jun N-terminal kinase (JNK) and S6 kinase (S6K). To investigate how a lipopolysaccharide (LPS)-mediated inflammatory response may modulate pathways implicated in insulin resistance, we characterized the LPS-induced changes in key biomarkers. Administration of 0.06–4 mg/kg LPS to C57BL/6 mice stimulated increases in plasma levels of TNFα, IL-12p40, IL-6 and MCP-1 and in JNK activity as measured by phosphorylated c-Jun in fat. For the first time, we show that LPS induces S6K activity by up to 6.1-fold, as measured by the phosphorylation of S6 ribosomal protein in liver, and increases by up to 1.8-fold, plasma levels of the novel pro-inflammatory cytokine osteopontin which is implicated in the pathogenesis of insulin resistance. These novel findings suggest that LPS administration may form the basis of an acute *in vivo* pharmacodynamic model for therapies targeting multiple pathways implicated in insulin resistance.

Keywords: JNK; S6K; Lipopolysaccharide; Osteopontin; Biomarker; Insulin sensitizer

With the increase of food availability and the emergence of obesity as a major metabolic disorder, there is an urgent need to develop effective therapies against the resulting development of insulin resistance and type 2 diabetes [1]. Several pathways stimulated by nutrient excess and/or induction of inflammatory pathways including c-Jun NH_2-terminal kinase 1 (JNK1) [2] , inhibitor of nuclear factor-κβ kinase [3], novel protein kinase C isoforms [4] and ribosomal protein S6 kinase 1 (S6K1) [5], have been implicated in playing a role in the suppression of insulin receptor mediated signaling pathways and are the focus of a large number of research efforts.

Dysregulation of cytokine production in metabolically active tissues such as adipose and muscle, combined with inflammation resulting from macrophage infiltration into adipose tissue, has been associated with a decrease in insulin action. JNK is activated by obesity-induced ER stress, TNFα, and fatty acids [6]. Upregulation of JNK activity plays a key role in activating the activating protein-1 (AP-1) transcription factor, via phosphorylation of c-Jun. Of the three JNK isoforms, JNK1 is the central player in modulating insulin action. JNK1 phosphorylates IRS-1 on serine residues including Ser^{307}, reducing IRS-1 tyrosine phosphorylation and signaling capacity through the insulin receptor [7]. JNK1 activity is elevated in liver, muscle and adipose in obese mice [2] and in skeletal muscle from obese and type 2 diabetic humans, where it is correlated with increased phosphorylation of IRS-1 at Ser^{307} [8]. Moreover, JNK1 deficiency has also been shown to protect mice from the development of insulin resistance by enhancing insulin signaling and reducing adiposity in diet-induced and genetic models of obesity [2]. Thus, inhibition of JNK1 activity may be a good approach to ameliorate insulin resistance.

S6K1 plays a pleiotropic role including, regulation of pancreatic β-cell growth, protein synthesis, accumulation of lipid in adipose and suppression of fatty acid oxidation

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in muscle [5]. The mammalian target of rapamycin (mTOR)/S6K1 insulin signaling pathway modulates biological effects of nutrients, insulin and glucose. S6K1 knockout mice are protected against diet-induced obesity and development of insulin resistance. Furthermore, S6K1 knockout mice on a high-fat diet have reduced levels of phosphorylated Ser^{307} and Ser^{632} IRS1, resulting in attenuation of insulin resistance [9]. S6K1 is activated in the skeletal muscle, adipose tissue, and liver of fat-fed obese rats [10]. Moreover, the elevation in phosphorylation of IRS-1 on $Ser^{636/639}$ observed in liver and muscle of insulin resistant obese rats is reversed following treatment with the inhibitor of mTOR signaling, rapamycin [11]. It remains unclear whether the mTOR/S6K pathway plays a role in hepatic glucose metabolism in humans since Krebs et al. [12] were unable to observe an effect of rapamycin on endogenous glucose production under hyperaminoacidemic and hyperinsulinemic conditions [11]. However, rapamycin was found to inhibit the ability of hyperaminoacidemia and prandial-like hyperinsulinemia to increase S6K1 phosphorylation and serine phosphorylation of IRS-1 in human skeletal muscle, emphasizing the importance of the mTOR/S6K1 pathway in the regulation of nutrient-sensitive glucose uptake in man [12]. Thus interventions to prevent dysregulation of the nutrient signaling pathways may also provide targets of interest for the reduction of insulin resistance.

Along with the increasing focus on pathways involved in disruption of insulin signaling as targets for type 2 diabetes, there is a need for an acute *in vivo* model to study the relative pharmacokinetic/pharmcodynamic effects of compounds and compare the relative effects of targeting different kinases in the same model. Treatment of rats or mice with gram-negative bacterial lipopolysaccharide (LPS), has been used extensively as a model of severe infection in humans and animals [13,14]. LPS treatment induces genes involved in the immune, inflammatory and acute phase responses and has major metabolic effects, including induction of insulin resistance [15]. Here we describe, for the first time, the elevation of S6K1 activity and of plasma levels of osteopontin, a novel biomarker of insulin resistance, following LPS treatment in C57BL/6 mice. Furthermore, we expand on previously published data, by characterizing the increase in JNK activity in response to LPS, relative to changes in cytokines and stimulation of S6K activity.

Materials and methods

Reagents. Escherichia coli LPS was obtained from Sigma–Aldrich (St. Louis, MO). Kits for analysis of phospho c-Jun (Ser63), phospho S6 ribosomal protein (Ser 235/236), insulin, and cytokines in addition to Tris lysis buffer, protease and phosphatase inhibitors were from Mesoscale Discovery (MSD) (Gaithersburg, MD). Mouse osteopontin ELISA kit was purchased from R&D systems (Minneapolis, MN).

Animal experiments. All animal procedures described in this report complied with the Pfizer IACUC and animal care guidelines. Male C57BL/6 mice (7–8 weeks old) obtained from Jackson Laboratory (Bar Harbor, ME), were housed in groups of 3 per cage in a temperature-controlled room with a light/dark cycle from (6:30 AM–6:30 PM). Mice had free access to food (Formulab Diet #5001, PMI Nutrition, Brentwood, MO) and water. Mice were acclimated for approximately 1 week before study start and randomly assigned to treatment groups. On the day of study food was removed at 9 AM. Vehicle (saline) or LPS was administered intraperitoneally (i.p.) at 11 AM. At the indicated time points, mice were anesthetized using isoflurane, blood was sampled via cardiac-puncture and tissue samples were removed from liver and epididymal fat. Tissues were placed in 1.5 mL lysing matrix D tubes (MPBio, Solon, OH) and immediately frozen in liquid nitrogen.

For insulin tolerance tests, blood glucose was determined using a glucometer (OneTouch®; Lifescan, Milpitas, CA) via tail-nicking of mice. Mice were bled approximately 2 h after LPS injection ($T = 0$) and human insulin (Humulin; Eli Lilly, Indianapolis, IA) was administered at 0.3 U/kg in 0.9% saline containing 3% bovine serum albumin. Blood glucose was measured at indicated times. Changes in insulin sensitivity following the bolus of insulin were examined by calculating the linear slope of the fall in glucose (Ki) subsequent to insulin administration.

Ex vivo analysis. Blood was collected in heparinized tubes for generation of plasma for analysis of cytokines, osteopontin, glucose, or insulin. Glucose was measured using a clinical analyzer (ALFA Wassermann, West Caldwell, NJ). For tissue analysis, 1 ml of modified Tris lysis buffer (containing per 10 ml; 200 μl MSD protease inhibitor, 200 μl phosphatase Inhibitor I and II, 1 mM PMSF and 0.5 M NaCl) was added to the liver or fat samples and homogenization performed using a Fast-Prep homogenizer (Q-biogene, Irvine, CA) . Samples were subsequently centrifuged for 10 min at 4 °C at 13,000 r.p.m. Protein content of the resulting supernatant was determined using the BCA kit (Pierce Biotechnology, Rockford, IL). Fifty microgram of tissue protein was used for determination of JNK and 20 μg for S6 kinase activities. JNK and S6 kinase activities were measured by quantitation of phospho-c-Jun (Pc-Jun) and phospho-S6 ribosomal protein (pS6RP), respectively, per manufacturer's instructions.

Statistics. Data are presented as arithmetic mean ± s.e.m. Statistical differences between treatment groups and vehicle were compared by unpaired students *t*-test, or one-way analysis of variance (ANOVA) followed by the Tukeys test for least significant differences (Graphpad PRISM, San Diego, CA). Results were considered significant at $P \leqslant 0.05$.

Results and discussion

Increasing awareness is now being placed on inflammatory and nutrient pathways as important players in the pathogenesis of insulin resistance and development of type 2 diabetes. It is crucial to understand, how *in vitro* efficacy translates on a pharmacokinetic/pharmacodynamic level *in vivo*. As a potential acute model for use across multiple targets in the inflammatory pathway, we have characterized the induction of several biomarkers of interest using the inflammatory mediator, LPS.

LPS has previously been shown *in vitro* and *in vivo* to induce insulin resistance and the expression of numerous cytokines through Toll-like receptor 4 (TLR4) [16]. Amongst the cytokines elevated by LPS is tumor necrosis factor α (TNFα), an established activator of the JNK pathway [17]. Free fatty acids also activate JNK and mediate their effects on metabolism through TLR4 in adipocytes and macrophages [18]. LPS has previously been shown to stimulate Pc-Jun in liver, white adipose tissue, and muscle of C57BL/6 mice and ob/ob mice 2 h following administration [19]. Moreover, studies in murine macrophages have demonstrated that LPS mediated Type 1 interferon (IFN) production and nitric oxide production occurs in-part

through a pathway utilizing PI 3-kinase, mTOR and S6K. Administration of LPS to C57BL/6 mice at 4 mg/kg elevated Pc-Jun in epididymal fat at 1.5, 2, and 7 h post-dosing (27.5-fold ($P < 0.01$), 11.7-fold ($P < 0.01$) and 10-fold (ns) respectively compared to vehicle controls) with the maximal response observed at 1.5 h (Fig. 1A). LPS stimulation of Pc-Jun in liver was less pronounced and more variable than the response observed in fat (data not shown). Liver homogenates were used to study changes in S6K1 since the hepatic activity was found to be highest in liver relative to that in adipose, muscle and pancreas (G. Bhat, J. Herrera, P, Tran, unpublished observations). LPS produced a robust elevation of pS6RP in liver over the time course of the study (Fig. 1B), with maximum stimulation observed 2 h after LPS dosing (1.5 h, 7.3-fold ($P < 0.01$); 2 h, 12-fold ($P < 0.01$); and 7 h, 2.4-fold (ns) increase compared to vehicle control).

In order to characterize the relative stimulation of JNK and S6K activities more completely, the effects of increasing doses of LPS on biomarkers of insulin resistance were examined. In mice, LPS is known to induce hypoglycemia a few hours after injection, an effect thought to be stimulated by production of IL-1 [20]. At all doses used in the study (0.06–4 mg/kg), a significant decrease in glucose was observed 2 h after LPS injection (Fig. 2A). However,

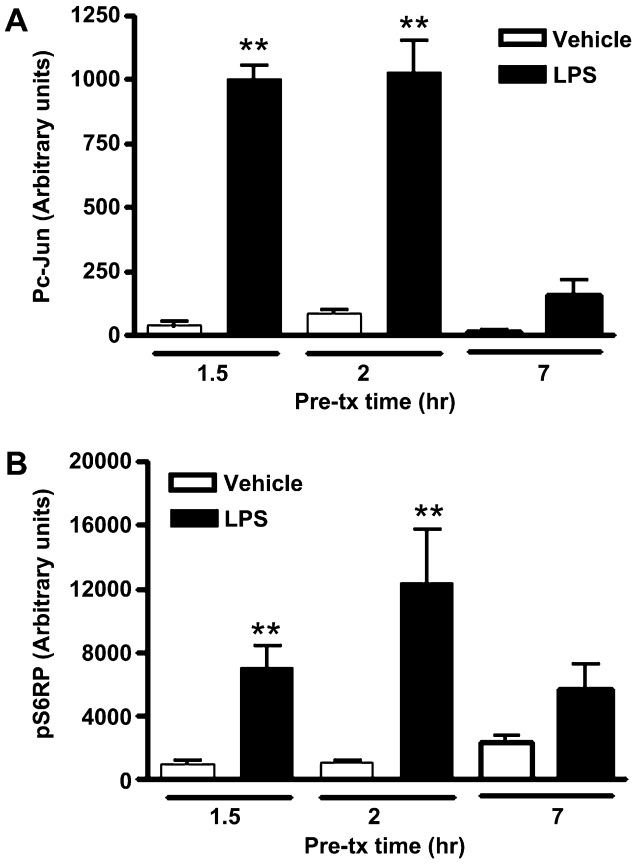

Fig. 1. Time-course of induction of (A) Pc-Jun (B) pS6RP following i.p. injection of 4 mg/kg LPS in C57BL/6 mice ($n = 5$ per group). Data represent the mean ± s.e.m. $^{**}P < 0.01$ vs vehicle treated group.

significant elevations in plasma insulin were only observed in mice that received 4 mg/kg LPS (mean ± s.e.m., vehicle 1.03 ± 0.33 ng/ml, 4 mg/kg LPS 2.63 ± 0.57 ng/ml; $P < 0.05$). To confirm an effect on whole-body insulin resistance, the change in glucose was determined following an insulin bolus in mice treated with 0.06 or 2 mg/kg LPS (Fig. 2B). Under the conditions used for the insulin tolerance test, where mice were conscious, there were no significant differences in whole-blood glucose prior to the administration of insulin. Mice that had received a control saline injection responded to the insulin bolus by an increase in glucose over the first 4 min, followed by a rapid drop in glucose over the subsequent 12 min. Mice that had received LPS responded to the insulin bolus by a continuous and more gradual decrease in glucose that reached a plateau after 12 min in mice treated with 0.06 mg/kg LPS and after 16 min in mice treated with 2 mg/kg LPS. Analysis of the linear slope of the decrease in glucose for all groups as a measure of insulin sensitivity (Ki), demonstrated that the Ki for mice treated with either the 0.06 or 2 mg/kg LPS was significantly greater than that for vehicle treated mice (Ki mean ± s.e.m.; vehicle −0.07 ± 0.0015 ($n = 6$), 0.06 mg/kg LPS −0.03 ± 0.009 ($n = 8$); [$P < 0.01$ vs vehicle], 2 mg/kg LPS −0.04 ± 0.008 ($n = 7$); [$P < 0.05$ vs vehicle] suggesting that both doses of LPS resulted in whole-body insulin resistance.

The dose-relationship between LPS and stimulation of signaling pathways implicated in attenuating insulin signaling was investigated. All doses of LPS tested significantly elevated production of monocyte chemotactic protein-1 (MCP-1), interleukin 12p40 (IL-12p40), TNFα and IL-6 ($P < 0.01$ compared to vehicle) (Fig. 3A). All doses of LPS also produced significant increases in Pc-Jun compared to vehicle (Fig. 3B), with 0.06 mg/kg producing a 4.3-fold elevation in Pc-Jun compared to vehicle ($P < 0.01$, $n = 4$) and 4 mg/kg LPS producing a 6-fold elevation in Pc-Jun ($P < 0.01$, $n = 6$). LPS-stimulated pS6RP levels did not appear to be dependent on the dose administered with 0.06 mg/kg LPS producing the largest increase in pS6RP of 6.1-fold compared to vehicle ($P < 0.01$, $n = 4$) (Fig. 3C). Further studies have also shown that doses of LPS as low as 0.01 mg/kg also produce a 5.5-fold elevation in S6K1 activity compared to vehicle (data not shown), suggesting that the dose-dependency of LPS-induced S6K1 activation may be different from that of LPS-stimulated JNK activity.

The mTOR/S6K1 pathway is known to be a key player in the negative effects of amino acids on insulin signaling. LPS signaling downstream of TLR4 is mediated through two different pathways, utilizing different Toll/IL-1R domain-interacting proteins, MyD88 and TIR domain-containing adapter inducing IFNβ (TRIF). Both pathways are important in the full response to LPS. Induction of cytokine gene expression in macrophages via activation of transcription factors NF-kB and AP-1, is mediated predominantly through MyD88 [21]. The non-MyD88 pathway is responsible for induction of Type 1 IFN, which is

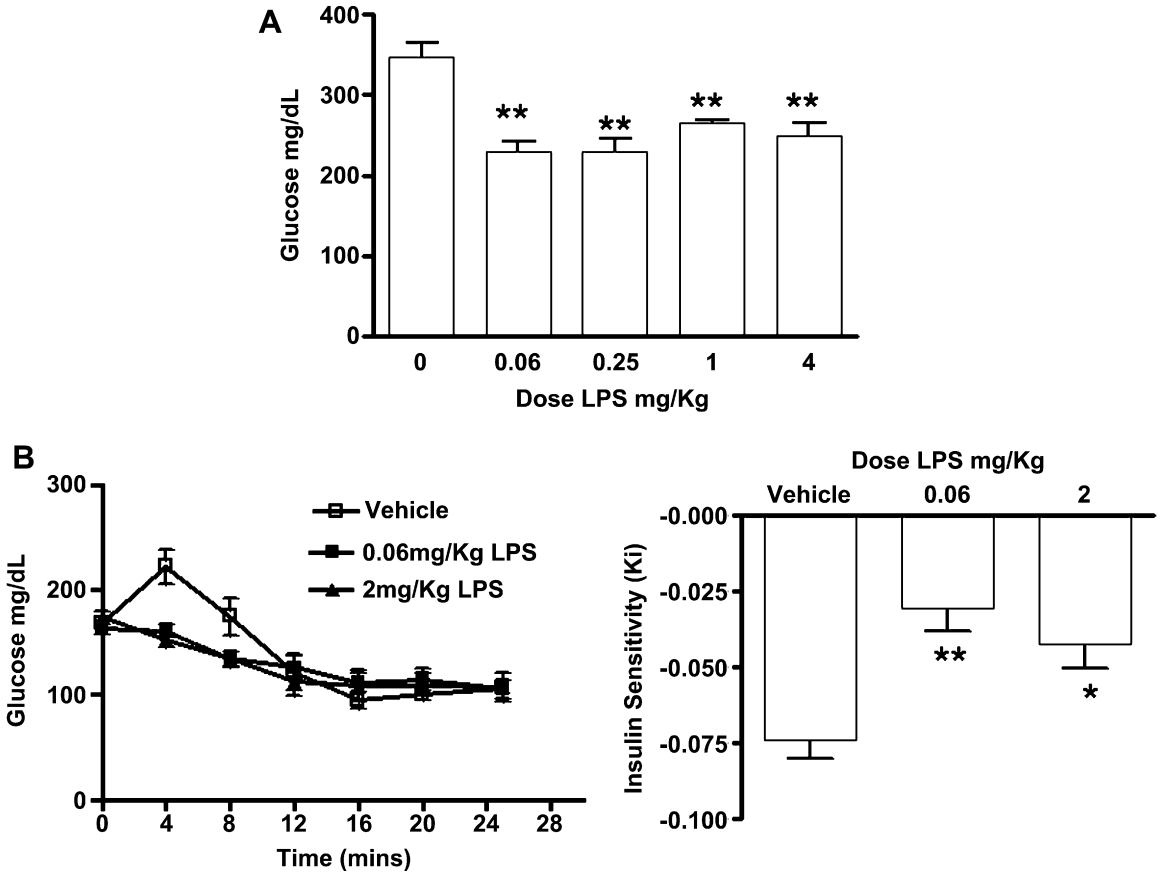

Fig. 2. Effects of LPS and insulin on glucose levels in C57BL/6 mice. (A) Suppression of plasma glucose 2 h subsequent to i.p. injection with LPS or saline ($n = 4$–6 animals/group). (B) Effect of LPS on insulin sensitivity of C57BL/6 mice. Animals were treated with indicated levels of LPS 2 h prior to insulin treatment (0.3 U/kg i.p.). Insulin sensitivity derived from the insulin tolerance test is shown as Ki, calculated as described in Materials and methods ($n = 6$–8 animals/group). Data represent mean ± s.e.m $^{**}P < 0.01$, $^{*}P < 0.05$ vs vehicle treated group.

associated with the later activation of NF-kB and AP-1 [21,22]. LPS-stimulated pathways leading to activation of S6K1 appear to be distinct from those leading to stimulation of JNK in macrophages, since inhibitors of PI 3-kinase or mTOR had little effect on LPS-stimulation of JNK activity. The difference in the ability and potency of LPS to stimulate S6K activity compared to JNK activity may therefore support previous reports suggesting that these kinases are activated via alternative pathways downstream of LPS.

Osteopontin is a multifunctional phosphoprotein secreted from various cell-types including epithelial cells and activated cells of the immune system [23,24]. It is implicated in the chemoattraction of monocytes and inflammatory gene expression [23,25]. Osteopontin levels are elevated in patients with type 2 diabetes [26] and correlate with the extent of coronary atherosclerosis [27]. Although there is no direct evidence of its role, the literature suggests that osteopontin may be involved in pathophysiology of insulin resistance and diabetic complications including cardiovascular disease. In the present study, mice that received 0.25 mg/kg LPS exhibited a 1.6-fold ($P < 0.01$, $n = 6$) increase in plasma osteopontin protein levels compared to mice receiving saline, which rose to a 1.8-fold increase in mice dosed with 4 mg/kg LPS ($P < 0.01$, $n = 6$) (Fig. 3D). Osteopontin binds to a cell-surface integrin-binding motif and its expression is induced by TNFα and IL-1β [23,24,28], while over-expression of AP-1 increases osteopontin promoter activity [29]. LPS may therefore induce osteopontin protein expression through the elevation of cytokines and/or elevation of JNK activity, further studies are necessary to fully characterize the mechanism. Another adipocytokine associated with insulin resistance, resistin, has previously been shown to be increased by LPS at the mRNA level in white adipose tissue (WAT), white blood cells of rats, and human blood monocytes [30].

In conclusion, our studies have demonstrated that LPS acutely elevates S6K1 activity *in vivo* and induces plasma protein expression of osteopontin. Moreover, characterization of the dose-dependent stimulation of JNK and S6K activity suggests that the kinases may be stimulated via different signaling pathways downstream of LPS. The study supports the use of low doses of LPS administration *in vivo* as an acute pharmacodynamic model for testing insulin sensitizers targeting the JNK and S6K pathways. The model also allows for functional effects on insulin sensitivity to be assessed. Indeed LPS-mediated elevation of TNFα was reduced in CD-1 mice using a small-molecule

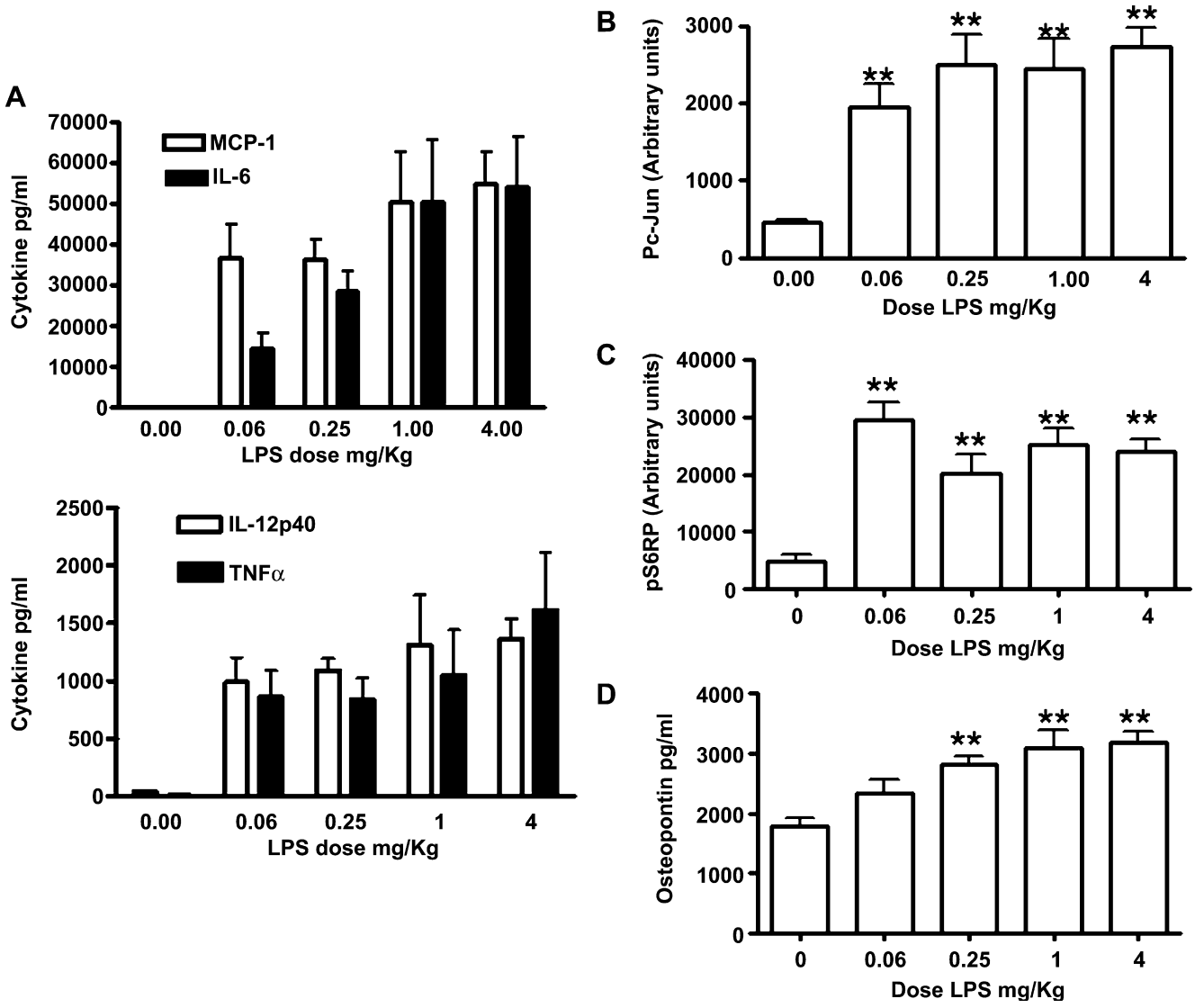

Fig. 3. Dose-dependent induction of biomarkers in LPS treated C57BL/6 mice. (A) Changes in cytokines IL-12p40, MCP-1, TNFα and IL-6 with administration of LPS or saline as indicated. All doses of LPS resulted in significant ($P < 0.01$) elevation in all cytokines measured compared to vehicle treated group. (B) Changes in Pc-Jun in epididymal fat with administration of LPS or saline as indicated. (C) Changes in pS6RP in liver with administration of LPS or saline as indicated. (D) Changes in osteopontin levels in plasma with administration of LPS or saline as indicated. Data represent mean ± s.e.m $^{**}P < 0.01$, vs vehicle treated group ($n = 4$–6 animals per group).

inhibitor of JNK [31], however inhibition of LPS-stimulated JNK activity was not analyzed. A recent report suggests that PPARα agonists, frequently used to treat dyslipidemia in patients with type 2 diabetes that are at high risk for cardiovascular disease, suppress osteopontin expression in macrophages and reduce osteopontin levels in plasma from patients with type 2 diabetes, via inhibition of AP-1 binding to the promoter [32]. It is therefore possible that inhibition of LPS-induced JNK activity may also lead to a reduction in osteopontin levels and therefore provide additional efficacy end-points for testing of small-molecules targeting JNK1.

In the current epidemic of obesity and type 2 diabetes, there is an increase in demand to develop novel and more effective therapies. As more potent and selective inhibitors for JNK and S6K1 are discovered, the low-dose LPS-induced insulin resistance model described here should be of value, not only as an *in vivo* screening method, but also as a means to identify potential biomarkers that may translate to chronic efficacy endpoints in rodent models and in the clinic.

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