Direct Effects of Locally Administered Lipopolysaccharide on Glucose, Lipid, and Protein Metabolism in the Placebo-Controlled, Bilaterally Infused Human Leg

Mads Buhl,* Ermina Bosnjak,* Mikkel H. Vendelbo, Jakob Gjedsted, Roni R. Nielsen, Thomas K.-Hafstrøm, Esben T. Vestergaard, Niels Jessen, Else Tønnesen, Andreas B. Møller, Steen B. Pedersen, Henriette Pilegaard, Rasmus S. Biensø, Jens O. L. Jørgensen, and Niels Møller

Medical Research Laboratories (M.B., E.B., M.H.V., J.G., R.R.N., T.K.-H., E.T.V., N.J., E.T., A.B.M., S.B.P., J.O.L.J., N.M.), Clinical Institute, Aarhus University, DK-8000, Aarhus, Denmark; Department of Pediatrics (M.B., T.K.-H.), Aarhus University Hospital, DK-8200 Aarhus, Denmark; Department of Endocrinology and Internal Medicine (E.B., M.H.V., E.T.V., N.J., A.B.M., S.B.P., J.O.L.J., N.M.), Aarhus University Hospital, DK-8000 Aarhus, Denmark; and Department of Cardiology (R.R.N.), Aarhus University Hospital, DK-8200 Aarhus, Denmark; and Department of Biology (H.P., R.S.B.), University of Copenhagen, DK-1165 Copenhagen, Denmark

Context: Accumulating evidence suggests that chronic exposure to lipopolysaccharide (LPS, endotoxin) may create a constant low-grade inflammation, leading to insulin resistance and diabetes. All previous human studies assessing the metabolic actions of LPS have used systemic administration, making discrimination between direct and indirect effects impossible.

Objective: We sought to define the direct, placebo-controlled effects of LPS on insulin resistance and protein and lipid metabolism in the infused human leg without systemic interference from cytokines and stress hormones.

Design: This was a randomized, placebo-controlled, single-blinded study.

Participants and Intervention: We studied 8 healthy volunteers with bilateral femoral vein and artery catheters during a 3-hour basal and 3-hour hyperinsulinemic-euglycemic clamp period with bilateral muscle biopsies in each period during infusion with saline and LPS.

Results: Overall, LPS perfusion significantly decreased leg glucose uptake, and during the clamp LPS decreased glucose arteriovenous differences (0.65 $\pm$ 0.07 mmol/L vs 0.73 $\pm$ 0.08 mmol/L). Net palmitate release was increased by LPS, and secondary post hoc testing indicated increased palmitate isotopic dilution, although primary ANOVA tests did not reveal significant dilution. Leg blood flows, phenylalanine, lactate kinetics, cytokines, and intramyocellular insulin signaling were not affected by LPS. LPS thus directly inhibits insulin-stimulated glucose uptake and increases palmitate release in the perfused human leg without detectable effects on amino acid metabolism.

Conclusions: These data strongly suggest that the primary metabolic effect of LPS is increased lipolysis and muscle insulin resistance, which, together with secondary insulin resistance, caused by systemic cytokine and stress hormone release may lead to overt glucose intolerance and diabetes. **(*J Clin Endocrinol Metab* 98: 2090–2099, 2013)**

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* M.B. and E.B. contributed equally to the study.

Abbreviations: AMPK, AMP-activated protein kinase; AS160, Akt substrate 160; AV, arteriovenous; FFA, free fatty acid; GM-CSF, granulocyte macrophage–colony-stimulating factor; GS, glycogen synthase; INF-γ, interferon-γ; LPS, lipopolysaccharide; PDH, pyruvate dehydrogenase; SA, specific activity.

Endotoxin (lipopolysaccharide [LPS]), a constituent of the outer membrane of the cell wall of Gram-negative bacteria, has the capacity to generate acute and chronic inflammation, leading to immune cell activation and cytokine release (1). LPS is a mediator of Gram-negative sepsis, and elevated endotoxin levels are found in up to 78.3% of the patients with severe sepsis or septic shock (2). Furthermore, accumulating evidence suggests that chronic overexposure to LPS, presumably derived from gut microbiota, in susceptible individuals may create a constant low-grade inflammation, leading to insulin resistance and eventually overt diabetes (3, 4). Animal studies have shown that prolonged exposure to LPS initiates obesity and insulin resistance (5), and human studies have revealed that increased LPS activity is associated with dyslipidemia, insulin resistance, obesity, and chronic inflammation and that these abnormalities may relate to decreased high-density lipoprotein elimination of LPS (6).

In this context, the metabolic actions of LPS are seminal. LPS binds to Toll-like receptor 4 and initiates signaling events, which include activation of cytokines (7). The fact that LPS induced insulin resistance and increased lipolysis in humans and rodents after a latency of 2 to 3 hours has been well described, although an early period with increased insulin sensitivity may be observed (8–11). Furthermore, it has been reported that LPS administration to humans increases whole-body protein breakdown, presumably in muscle (10). It is also clear that LPS administration creates generalized release of proinflammatory cytokines and that these, in particular TNF-α, mimic the response to infection and induce insulin resistance and increased lipolysis (12). Finally, LPS in combination with a number of cytokines activates the hypothalamo-pituitary axis and stimulates the release of stress hormones, such as epinephrine, glucagon, cortisol, and GH into the blood (13–15); all of these counterregulatory stress hormones generate insulin resistance (14–17). These effects may be mediated by Akt and Akt substrate 160 (AS160) signaling events in muscle cells (18). Other possible effectors include glycogen synthase (GS), a key enzyme in glycogen synthesis, and AMP-activated protein kinase (AMPK) (19). Finally, elevated plasma free fatty acid (FFA) concentrations have been suggested to down-regulate pyruvate dehydrogenase (PDH) activity and thus potentially contribute to insulin resistance (20). Thus, LPS generates release of both cytokines and stress hormones, and it is uncertain to what extent the metabolic actions of LPS are intrinsic or caused by cytokines or by stress hormones in humans.

The present study was designed to define the direct metabolic effects of LPS in human muscle. Because all previous human studies assessing the metabolic actions of LPS have used systemic administration, making discrimination between direct and indirect effects impossible, we simultaneously infused LPS directly into the femoral artery of one leg and compared our observations with those for the saline-infused contralateral leg (t = 0–360 min).

Subjects and Methods

Subjects

Eight healthy male volunteers, 27.5 ± 1.0 years of age and body mass index of 23.4 ± 0.5 kg/m^2, were included in the study after oral and written informed consent was obtained.

Unremarkable medical pasts were confirmed during a medical interview, and results of normal blood test screening and physical examination were present. The study was approved by the Central Denmark Region Ethics Committee (M-2007-0265), in accordance with the Declaration of Helsinki.

The subjects reported to the laboratory at 7:00 AM after an overnight fast. Vigorous physical exercise was not allowed for 2 days before participation in the study.

Leg model

As described previously (21), the Seldinger technique was used to insert catheters into the femoral artery and vein of both legs under local anesthesia. Femoral arteries and veins were visualized directly using ultrasonography. One leg received LPS and the other saline in a single-blind random (right vs left) manner from t = 0 to 360 minutes.

The proximal lumina of double-lumen arterial catheters were used for infusion of either LPS (USP Endotoxin [lot G3E069]; The United States Pharmacopeial Convention, Inc., Rockville, Maryland) or placebo (isotonic saline), respectively, in a single-blind randomized manner. LPS was diluted in isotonic saline and was, administered continuously over 360 minutes at an infusion rate of 0.025 ng/kg/h. Blood samples were taken from the arterial catheter infusing the placebo and from both venous catheters. One catheter was placed in a cubital vein for infusion of saline, metabolite tracers, insulin, amino acids, and glucose.

Femoral arterial blood flow was measured using Vivid e (GE Healthcare, Chalfont St Giles, Buckinghamshire, United Kingdom). In brief, angle-corrected pulsed-wave Doppler (blood flow velocity) measurements were performed at the tip of the catheter. The diameters of the arteries were measured using the two-dimensional images, and the flow was estimated by calculating the mean flow from 3 measurements (each based on 10 pulse waves) during the last 20 minutes of the basal and the clamp period.

Hyperinsulinemic-euglycemic clamp

The study consisted of a 180-minute basal period ("basal"), followed by a 180-minute hyperinsulinemic-euglycemic clamp period ("clamp"). Infusion rates of insulin (Insulin Actrapid; Novo-Nordisk, Copenhagen, Denmark) were 1.0 mU/kg/min iv. Systemic plasma glucose was clamped at 5 mmol/L by variable infusion of 20% glucose, and arterial plasma glucose concentrations were measured at least every 10 minutes (Beckman Instruments, Palo Alto, California). During the clamp, amino acids were infused (Glavamin 22.4 g N/L; Fresenius Kabi AB, Uppsala,

Sweden; infusion rate: 1.056 ml/kg/h) to avoid a decrease in amino acid levels.

Phenylalanine and palmitate kinetics

Albumin-bound [9,10-^{3}H]palmitate (GE Healthcare) and [^{15}N]phenylalanine (Cambridge Isotope Laboratories, Andover, Massachusetts) were used as metabolite tracers. Palmitate was infused (infusion rate, 0.3 μCi/min) from $t = 120$ to 180 minutes and again from $t = 300$ to 360 min. Blood samples for measurements of palmitate concentration and specific activity (SA) were obtained before infusion and after 40, 50, and 60 minutes of the infusion period. Plasma palmitate concentration and SA were determined by HPLC using [$^2H_{31}$]palmitate as an internal standard (22). Palmitate was analyzed in triplicate during steady state. Regional palmitate net balances were estimated using blood flow and SA from arterial and venous samples and calculated as described previously (23).

A primed continuous infusion of [^{15}N]phenylalanine (prime, 0.75 mg/kg; infusion rate, 0.75 mg/kg/h) was started at $t = 0$ and maintained until termination of the study. Enrichments of [^{15}N]phenylalanine were measured by gas chromatography–mass spectrometry as their *t*-butyldimethylsilyl ether derivates under electron ionization conditions, and the concentration of phenylalanine was measured (for calculation of regional amino acid kinetics) using L-[$^2H_8$] phenylalanine as an internal standard (24).

Leg protein breakdown, represented by phenylalanine rate of appearance, and muscle protein synthesis rate, represented by phenylalanine rate of disappearance, were calculated:

Phenylalanine balance (PheBal) was calculated as follows using Fick's principle:

$$\text{PheBal} = (\text{Phe}_A - \text{Phe}_V) \times F$$

where Phe_A and Phe_V are arterial and venous phenylalanine concentrations and F is blood flow in the leg. Regional phenylalanine kinetics was calculated, using the equations described by Nair et al (24). Leg protein breakdown, represented by phenylalanine rate of appearance (R_a Phe), was calculated as follows (25):

$$R_a\,\text{Phe} = \text{Phe}_A = [(\text{PheE}_A/\text{PheE}_V) - 1] \times F$$

where PheE_A and PheE_v represent phenylalanine isotopic enrichment in arteries and veins. The local rate of disappearance, which represents the muscle protein synthesis rate, was calculated as follows:

$$R_d\,\text{Phe} = \text{PheBal} + R_a\,\text{Phe}$$

Plasma [^{15}N]phenylalanine enrichment and [9,10-^{3}H]palmitate SA were at a plateau at the time of sampling (data not shown).

Serum FFAs were determined by a colorimetric method using a commercial kit (Wako Chemicals, Neuss, Germany), and lactate concentrations were determined by an automated analyzer (Cobas b221; Roche, Hvidovre, Denmark).

Muscle biopsies and Western blotting

Muscle biopsy samples were obtained simultaneously under local anesthesia with Bergström biopsy needles from both lateral vastus muscles at $t = 120$ minutes and $t = 210$ minutes. Biopsy samples were cleaned for visual blood immediately, snap-frozen in liquid nitrogen, and stored at −80°C until analyzed.

Muscle biopsy samples were homogenized in an ice-cold buffer containing 20 mM Tris-HCl, 50 mM NaCl, 50 mM NaF, 5 mM tetrasodium pyrophosphate, 270 mM sucrose, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM glycerophosphate, 2 mM dithiothreitol, 50 μg/mL soybean trypsin inhibitor, 4 μg/mL leupeptin, 100 μM benzamidine, and 500 μM phenylmethylsulfonylfluoride (pH 7.4), and samples were rotated for 60 minutes at 4°C. Insoluble materials were removed by centrifugation at 16,000*g* for 20 minutes at 4°C.

Western blot analyses were used to assess protein and phosphorylation levels of various proteins. Antibodies to Akt (no. 3063), AS160 (no. 2447), GS (no. 3886), and AMPK (no. 2532) and phosphospecific antibodies Akt Thr^{308} (no. 9275), Akt Ser^{473} (no. 9271S), AS160 Thr^{642} (no. 4288S), AS160 Ser^{588} (no. 8730S), PAS (no. 9611), GS Ser^{641} (no. 3891), and AMPK Thr^{172} (no. 2531) were from Cell Signaling Technology (Danvers, Massachusetts). Phosphorylation of PDH-E1α site 1 (Ser^{293}) and site 2 (Ser^{300}) and protein expression of PDH-E1α (antibodies kindly provided by Professor G. D. Hardie, University of Dundee, Dundee, Scotland) were measured in muscle samples by SDS-PAGE and Western blotting. Proteins were visualized by BioWest enhanced chemiluminescence (Pierce Chemical, Rockford, Illinois) and quantified using a UVP BioImaging System (UVP, Upland, California). Quantifications of protein phosphorylation are expressed as a ratio of total protein expression measured on the same membranes.

Cytokine measurements

Samples were diluted 1:2 and cytokine (granulocyte macrophage–colony-stimulating factor [GM-CSF], interferon-γ [INF-γ], IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and TNF-α) concentrations in plasma were quantified using a Human Ultrasensitive Cytokine 10-Plex Panel (Invitrogen, Carlsbad, California). All cytokine measurements were run in duplicate (Luminex 100 Bioanalyzer; Luminex Corp, Austin, Texas). According to the manufacturer's information sheet, interassay coefficients of variation were between 4.4% and 8.6%.

Quantitative PCR

RNA isolation and quantitative PCR analysis were performed using standard protocol. See Supplemental Methods, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

Statistics

Data are presented as means ± SEM. Statistical analysis was performed using 2-way repeated-measures ANOVA for the primary analysis and paired *t* tests for post hoc analysis, as outlined in our original protocol. All ANOVA results refer to an overall main effect of LPS vs placebo (we only saw a significant insulin/LPS interaction as regards Akt Ser^{473} signaling); in addition, we have given *P* values for post hoc paired *t* tests comparing the 2 legs (the effect of LPS vs saline) under a single treatment condition (basal or clamp). Normal distribution was assessed by inspection of QQ plots, and Wilcoxon signed rank tests were used to test nonnormally distributed data.

Results

The characteristics of the volunteers and arterial hormone levels are given in Table 1. Because of the nature of the

model, arterial hormone and metabolite levels were similar.

Basal (Tables 1–3 and Figures 1–3)

Arterial glucose concentrations were 5.3 ± 0.1 mmol/L, and blood flows were not different between the 2 legs during the basal period (Table 2). Overall, 2-way ANOVA for repeated measurements revealed a main LPS effect to decrease glucose differences ($P = .015$) (Figure 1), although specific basal glucose differences only reached borderline significance ($P = .095$) when tested separately by post hoc *t* tests (LPS vs saline) Arterial concentrations of free fatty acids were 0.473 ± 0.043 mmol/L. Overall 2-way ANOVA for repeated measurements showed a main LPS effect ($P = .004$) to decrease palmitate arteriovenous (AV) differences (indicating increased release: −0.50 ± 7.32 μmol/L [LPS] vs 8.88 ± 6.29 μmol/L [placebo]) (Table 2). Although ANOVA showed no overall effect on palmitate dilution, post hoc paired *t* tests (comparison of LPS vs saline within the basal condition) showed significant dilution (palmitate specific activity: 2.0 ± 0.23 cpm/μmol [LPS] vs 2.2 ± 0.26 cpm/μmol [placebo], $P < .03$) compatible with increased lipolysis.

Calculated rates for palmitate and phenylalanine appearance and disappearance were unaltered (all $P > .2$). Lactate release tended to be increased by regional LPS infusion ($P = .081$), whereas skeletal muscle net protein balance (measured with phenylalanine) and rates of proteolysis and synthesis were similar in both legs.

Clamp (Tables 1–3 and Figures 1–3)

Arterial glucose concentrations were clamped at 5.0 ± 0.1 mmol/L, and steady-state glucose infusion rates during the last 30 minutes of the clamp were recorded.

Overall 2-way ANOVA for repeated measurements revealed a main LPS effect to decrease glucose differences ($P = .015$) (Figure 1 and Table 2), although glucose AV differences during the clamp only reached borderline significance ($P = .068$) when tested separately by post hoc *t* tests (comparison of LPS vs saline within the clamp condition).

Arterial concentrations of free fatty acids were 0.081 ± 0.034 mmol/L. ANOVA showed an overall effect of LPS, and in parallel with the basal state, palmitate AV differences were increased (2.50 ± 4.61 μmol/L [LPS] vs 10.25 ± 4.07 μmol/L [placebo], $P = .023$) (Table 2), but specific activities and rates of appearance and disappearance remained unaltered. Lactate release was not affected, and skeletal muscle net protein balance (measured by phenylalanine) and rates of proteolysis and synthesis were similar under both conditions.

Cytokines (Table 3)

Cytokine levels (GM-CSF, INF-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and TNF-α) were similar in both legs (Table 3), and mRNA expression (Supplemental Table 4) of a number of cytokines was not substantially affected. Normally distributed data were tested by paired *t* tests, and in the absence of normal distribution, Wilcoxon signed rank tests were used for analysis in the basal and clamped state (LPS vs saline).

Biopsies

To assess whether insulin signaling was impaired in the LPS-treated leg, we measured phosphorylation of Akt. Phosphorylation of Thr^{308} and Ser^{473} on Akt responded to insulin stimulation, and Ser^{473} was decreased by 25% in the placebo leg ($P = .016$) (Figure 3, A and B). This difference was not present downstream of Akt at the level of AS160. Phosphorylation of AS160 was detected with antibody against phosphorylated Akt substrate sites (PAS) and specific antibodies against AS160 Thr^{642} and AS160 Ser^{588}, and all sites responded to insulin with no effect of LPS (Figure 3, C, D, and E). Furthermore, dephosphorylation of glycogen synthase by insulin was equal in the 2 legs (Figure 3F). Glycolytic pathways were assessed with examination of Ser^{293} (site 1) and Ser^{300} (site 2) on PDH-E1α. During the clamp there was a trend toward reduced

Table 1. Characteristics of the Volunteers and Arterial Hormone Levels

	0 min	Basal	*P* Value[a]	Clamp	*P* Value[a]
Mean BP, mm Hg	91 ± 3.3	90 ± 3.3	.178	87 ± 3.7	.153
HR, beats/min	66.3 ± 4.4	71 ± 3.3	.21	75.8 ± 4.1	.016
Insulin, pmol/L	41.6 ± 4.9	30.9 ± 1.9	.06	304.9 ± 19.3	<.001
Glucagon, pg/mL	61.9 ± 10.3	39.9 ± 5.2	.053	161.9 ± 27.8	.008
Cortisol, ng/mL	179 ± 22.5	135 ± 16.7	.063	111.9 ± 16.7	.051
GH, ng/mL	0.6 ± 0.2	1.5 ± 0.8	.241	4.3 ± 2.5	.192
Age, y	27.5 ± 1.0				
BMI, kg/m^2	23.4 ± 0.5				

Abbreviations: BMI, body mass index; BP, blood pressure; HR, heart rate.

[a] *P* values were calculated by paired *t* tests, comparing with time 0 minutes (start of experiments).

Table 2. Regional AV Balances and Metabolism

	Basal		Clamp		*P* Value[a]		
	LPS	Placebo	LPS	Placebo	Main LPS vs Placebo Effect	Main Insulin/ Time Effect	LPS Insulin/ Time Interaction
Blood flow, mL/min	409 ± 42	402 ± 31	383 ± 38	367 ± 42	.364	.443	.845
Glucose AV difference, mmol/L	0.02 ± 0.02	0.06 ± 0.02	0.65 ± 0.07	0.73 ± 0.08	.015	<.001	.450
Palmitate arterial concentrations, μmol/L		178.8 ± 15.9		40.6 ± 10.2			
Palmitate venous concentrations, μmol/L	179.3 ± 12.1	169.9 ± 13.2	38.1 ± 7.7	30.4 ± 6.7	.004	<.001	.698
Palmitate AV difference, μmol/L	−0.50 ± 7.32	8.88 ± 6.29	2.50 ± 4.61	10.25 ± 4.07	.004	.739	.698
Palmitate SA, cpm/μmol	2.0 ± 0.23	2.2 ± 0.26	5.6 ± 1.4	6.8 ± 1.6	.409	.020	.500
Lactate AV difference, mmol/L	−0.08 ± 0.03	−0.04 ± 0.03	−0.09 ± 0.03	−0.09 ± 0.04	.378	.470	.333
Phe arterial concentrations, mg/L		7.06 ± 0.42		14.10 ± 0.84			
Phe venous concentrations, mg/L	7.85 ± 0.54	7.84 ± 0.59	13.01 ± 0.72	12.95 ± 0.56	.604	<.001	.867
Phe AV difference, mg/L	−0.79 ± 0.16	−0.78 ± 0.23	1.09 ± 0.19	1.15 ± 0.34	.604	.002	.867
Phe arteriovenous enrichment	0.027 ± 0.005	0.025 ± 0.004	0.008 ± 0.001	0.008 ± 0.002	.601	.005	.179

Abbreviation: Phe, phenylalanine tracer.

[a] *P* values were calculated by 2-way repeated-measures ANOVA.

phosphorylation of the PDH site 1 (main effect, $P = .06$), whereas site 2 did not change. PDH phosphorylation was unaffected by LPS treatment (Figure 3, G and H). AMPK phosphorylation remained stable under all circumstances (Figure 3I).

Discussion

This study was designed to define the direct metabolic effects of LPS in human muscle and to test whether local placebo-controlled leg infusion of LPS directly induces insulin resistance and affects protein and lipid metabolism. The main outcome of the study is that LPS directly increases net palmitate release and induces insulin resistance in terms of decreased glucose AV differences during a systemic hyperinsulinemic-euglycemic clamp. Post hoc paired *t* tests (LPS vs saline) showed increased palmitate dilution in the basal state compatible with increased basal lipolysis. Furthermore, intramyocellular insulin signaling, amino acid metabolism, and release of lactate and cytokines in general remained unaffected. We only observed minor changes in local leg cytokine release after LPS exposure, suggesting that the cytokine surges after systemic LPS exposure are derived from other sites.

Previous studies have reported that LPS, after a latency of 2 to 3 hours, induces hyperglycemia, insulin resistance, and increased lipolysis in humans, but no studies have examined the local effects of LPS on muscle glucose uptake in humans (8). However, classic venous catheterization studies have shown that during a high-dose glucose clamp approximately 85% of whole-body glucose disposal is accounted for by striated muscle (26), so it can be inferred that LPS directly or indirectly severely impairs muscle glucose uptake. Our study revealed a 10% to 15% decrease in leg glucose uptake after 6 hours of local LPS infusion, compatible with the notion that LPS may contribute directly to muscle insulin resistance. The mechanisms whereby LPS leads to a decrease in muscle glucose uptake are not clear. The difference in insulin sensitivity was not

Table 3. Cytokine Plasma Concentrations

		Basal			Clamp		
	0 Min	LPS	Placebo	*P* Value[a]	LPS	Placebo	*P* Value[a]
TNF-α, pg/mL							
Arterial[b]	80.12 ± 12.69		61.62 ± 11.81	.181		74.79 ± 12.44	.743
Venous[c]		71.81 ± 14.9	79.47 ± 14.19	.426	69.24 ± 12.05	78.26 ± 11.55	.541
AV difference[d]		−10.2 ± 15.1	−17.9 ± 12.7	.426	5.5 ± 15.2	−3.5 ± 8.8	.541
INF-γ, pg/mL							
Arterial[b]	46.81 ± 12.57		36.67 ± 9.50	.039		35.64 ± 8.21	.383
Venous[c]		41.91 ± 10.59	43.63 ± 10.17	.706	41.42 ± 10.19	36.84 ± 8.47	.410
AV difference[d]		−5.2 ± 8.5	−7.0 ± 6.29	.706	−5.8 ± 4.7	−1.2 ± 5.7	.410
IL-1β, pg/mL							
Arterial[b]	5.95 ± 1.96		5.94 ± 1.56	.607		6.44 ± 1.67	.578
Venous[c]		5.05 ± 1.38	6.25 ± 1.80	.076	5.95 ± 1.66	6.26 ± 1.64	.589
AV difference[d]		0.9 ± 0.9	−0.3 ± 1.06	.132	0.5 ± 0.3	0.2 ± 0.5	.589
IL-2, pg/mL							
Arterial[b]	17.69 ± 5.32		16.72 ± 6.38	1.000		17.64 ± 7.36	1.000
Venous[c]		17.50 ± 6.94	18.30 ± 6.90	.492	16.96 ± 5.84	18.21 ± 7.38	.313
AV difference[d]		−0.8 ± 2.0	−1.6 ± 1.63	.492	0.7 ± 1.9	−0.6 ± 1.1	.571
IL-4, pg/mL							
Arterial[b]	143.31 ± 44.94		123.22 ± 50.22	.352		125.91 ± 46.20	.354
Venous[c]		117.85 ± 30.75	128.33 ± 38.08	.507	119.68 ± 46.77	117.50 ± 25.10	.383
AV difference[d]		5.4 ± 27.4	−5.1 ± 29.70	.507	6.2 ± 14.0	8.4 ± 24.4	.939
IL-5, pg/mL							
Arterial[b]	7.84 ± 1.28		5.75 ± 0.88	.039		5.91 ± 0.88	.065
Venous[c]		6.80 ± 1.29	6.88 ± 0.84	.528	6.86 ± 1.35	6.63 ± 1.15	.842
AV difference[d]		−1.1 ± 1.1	−1.1 ± 0.64	.915	−1.0 ± 0.8	−0.7 ± 1.1	.842
IL-6, pg/mL							
Arterial[b]	38.05 ± 10.41		36.48 ± 9.74	.461		40.98 ± 6.73	.250
Venous[c]		34.10 ± 8.13	40.01 ± 8.10	.221	52.08 ± 11.17	52.80 ± 9.09	.930
AV difference[d]		2.4 ± 7.7	−3.5 ± 5.73	.221	−11.1 ± 8.2	−11.8 ± 6.0	.930
IL-8, pg/mL							
Arterial[b]	50.80 ± 14.40		46.97 ± 17.45	.383		57.46 ± 17.52	.311
Venous[c]		49.10 ± 11.34	47.65 ± 12.72	.711	61.41 ± 16.54	51.55 ± 7.84	.341
AV difference[d]		−2.1 ± 8.4	−0.7 ± 7.18	.711	4.0 ± 6.4	5.9 ± 11.5	.341
IL-10, pg/mL							
Arterial[b]	31.38 ± 9.46		31.11 ± 11.33	.633		40.45 ± 10.73	.132
Venous[c]		29.79 ± 8.63	30.33 ± 8.83	.845	44.96 ± 11.56	39.02 ± 8.53	.255
AV difference[d]		1.3 ± 5.0	0.8 ± 4.81	.845	−4.5 ± 4.3	1.4 ± 5.2	.255
GM-CSF, pg/mL							
Arterial[b]	77.11 ± 8.89		59.04 ± 9.44	.146		70.08 ± 12.15	.549
Venous[c]		70.98 ± 11.72	73.96 ± 10.31	.687	68.21 ± 9.55	70.18 ± 12.18	.874
AV difference[d]		−11.9 ± 10.3	−14.9 ± 9.72	.687	1.9 ± 12.6	−0.1 ± 8.1	.874

None of the main ANOVA comparisons (LPS effect, insulin effect, and LPS × insulin interaction) were significant, leading to the presentation of only within-condition comparisons.

[a] *P* values were calculated by paired *t* tests, and in the absence of normal distribution, Wilcoxon signed rank tests were used for analysis in the basal and clamped state. *P* values represent LPS leg vs placebo leg.

[b] Cytokine arterial plasma concentrations measured in the placebo leg; *P* values were calculated by paired *t* test and Wilcoxon signed rank test, comparing with time 0 minutes (start of experiments).

[c] Cytokine venous plasma concentrations.

[d] Cytokine AV differences.

reflected in intramyocellular insulin signaling to GLUT4 translocation, glycogen synthesis, or PDH activation, and we did not detect any difference in local cytokine expression or local release, which could explain the finding. Some studies have reported that FFAs induce inhibition of proximal insulin signaling through insulin receptor substrate-1 (IRS-1) and reduced Akt phosphorylation, leading to insulin resistance (27, 28). On the other hand, several subsequent reports have demonstrated that insulin sensitivity in skeletal muscle can be modulated by FFAs despite normal phosphorylation of Akt (29, 30), suggesting that either direct substrate competition or alternative mechanisms downstream of Akt may cause insulin resistance.

Our study clearly suggests that the direct effects of LPS to induce muscle insulin resistance are not dependent on

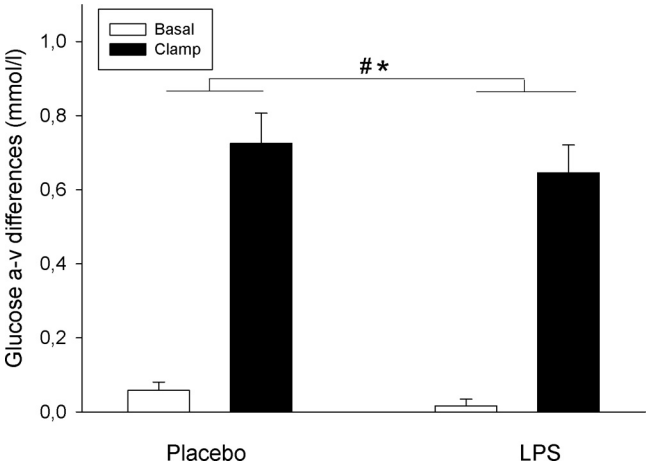

Figure 1. Glucose AV differences calculated from raw AV balances. White bars, LPS leg; black bars, placebo leg. Mean values from triplicate sampling at times 160, 170, and 180 minutes (basal) and 340, 350, and 360 minutes (clamp) are shown. #$P = .015$, LPS vs placebo (LPS main effect vs placebo by 2-way ANOVA for repeated measures). *$P < .001$, basal vs clamp (main insulin effect vs basal by 2-way ANOVA for repeated measures).

suppression of the intramyocellular insulin signal. In fact, in vitro cell culture studies in cardiomyocytes report that LPS, if anything, activates the phosphatidylinositol 3-kinase/Akt signaling pathway (31). Finally, although we did not detect increased release or expression of a number of cytokines measured, we cannot entirely exclude the possibility that local cytokine activation contributes to the observed metabolic effects of LPS in the leg.

Another possibility is that the observed muscular insulin resistance relates directly to increased FFA levels. Since the original conceptualization by Randle et al (32), a number of studies have confirmed the capacity of FFAs to reduce muscle glucose uptake and oxidation. Many of these studies have reported that this impairment of insulin sensitivity is not associated with reduced insulin signaling (27). Our study showed increased palmitate release and increased palmitate dilution in the LPS infused leg, indicating increased lipolysis, which may contribute to insulin resistance. Calculated values for palmitate rates of appearance were not significantly affected, probably reflecting a type 2 error relating to large variability in local leg blood and plasma flows. Human studies have shown increased femoral vein release of free fatty acids 2 to 6 hours after a systemic LPS bolus in healthy volunteers studied once (10, 11), and investigations of the direct lipolysis effect of endotoxin in rodents and in isolated primary adipocytes have reported increased lipolysis and activity and protein levels of hormone-sensitive lipase and adipose triglyceride lipase (11). It is uncertain how LPS stimulates lipolysis, but it is of interest that insulin dampens the oxidative, nitrosative, and inflammatory stress response to systemic LPS (33) and, as shown in our study, also inhibits lipolysis. Furthermore, systemic infusion studies have shown that LPS generates local inflammation in adipose tissue, consistent with a state of low-grade inflammation (34). In this context, it is also of importance to point out that although local muscle lipolysis may be of some significance, most fatty acids released during LPS exposure in all probability are derived from the adipose tissue reservoir and that it remains uncertain to what extent this efflux may directly affect local muscle insulin sensitivity. In the context of diabetes and chronic low-grade inflammation, it appears more likely that fatty acids exert their diabetogenic action at the whole-body level. As pointed out by Boden (35), circulation of FFAs is one of the very few, if not the only, possible physiological link, which has been shown to (1) be elevated in people with obesity and type 2 diabetes (36) and (2) cause insulin resistance in a dose-dependent manner (29).

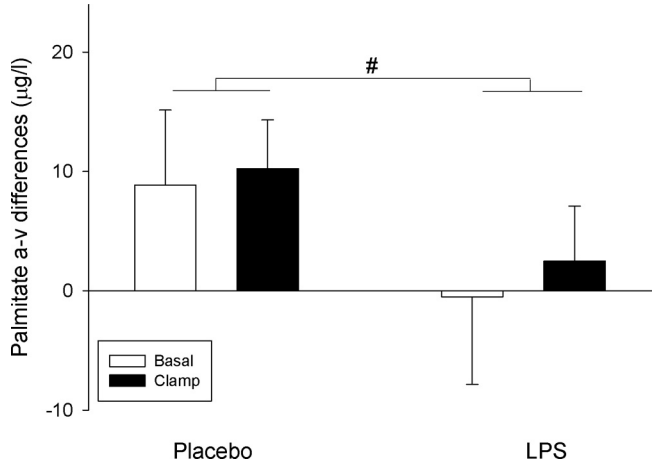

Figure 2. Palmitate AV differences. White bars, LPS leg; black bars, placebo leg. Mean values from triplicate sampling at times 160, 170, and 180 minutes (basal) and 340, 350, and 360 minutes (clamp) are shown. #$P = .004$, LPS vs placebo (LPS main effect vs placebo by 2-way ANOVA for repeated measures).

In humans, iv LPS injection increased muscle amino acid release (37) and decreased protein synthesis and breakdown (38). Our study, in which we gave LPS locally, did not reveal any evidence of altered amino acid metabolism and therefore suggested that the catabolic effects of LPS are not direct but rather are related to the systemic nature of exposure and release of secondary mediators such as cytokines or stress hormones.

In the current study, we introduced a new method to study the direct effects of LPS in the bilaterally perfused, placebo-controlled leg. The method has been validated and is well suited for investigations of metabolites and hormones with a short half-life and good penetration into muscle and adipose tissue (18). Previous studies have shown clear metabolic effects of a low-dose, 4-hour systemic 0.075 ng/h LPS infusion (13), so we infused one third (0.025 ng/kg/h) directly into the femoral artery in an at-

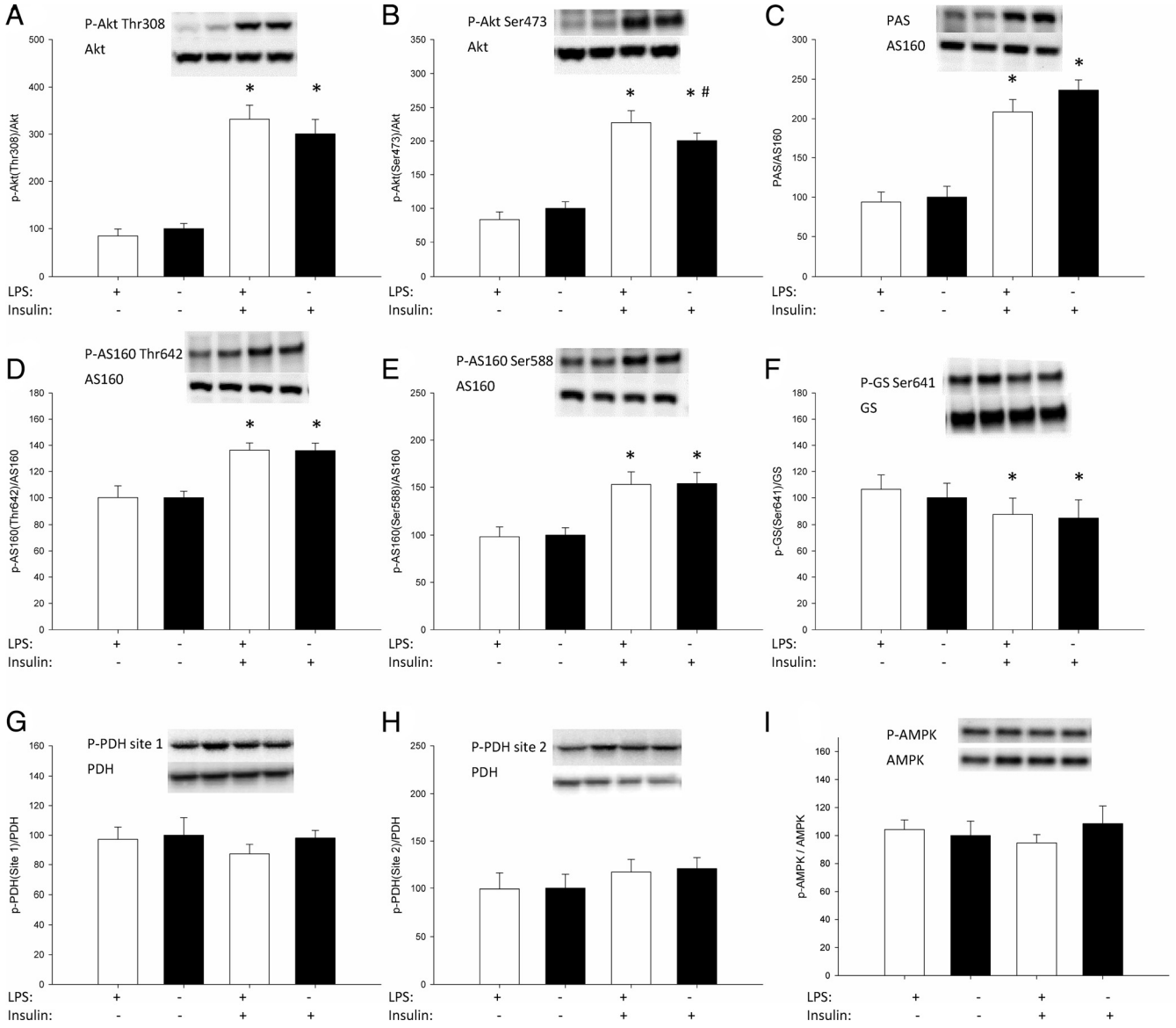

Figure 3. Intramyocellular signaling. White bars, LPS leg; black bars, placebo leg. Phosphorylation of Akt Thr^{308} (A), Akt Ser^{473} (B), PAS (C), AS160 Thr^{642} (D), AS160 Ser^{588} (E), GS Ser^{641} (F), PDH site 1 (G) and 2 (H) and AMPK Thr^{172} (I). *$P < .05$, basal vs clamp (main insulin effect vs basal by 2-way ANOVA for repeated measures). #$P < .05$, LPS vs placebo (LPS main effect vs placebo by 2-way ANOVA for repeated measures).

tempt to produce elevated local concentrations and minimize systemic effects. For technical reasons, we have not been able to accomplish reliable measures of LPS concentrations, but with a recorded blood flow of 400 ml/min (24 L/h), the infusion would give rise to a 0.003 ng/L increase of concentrations in the infused leg. We cannot exclude the possibility that cytokine or LPS spillover from the infused leg has affected systemic metabolism, as suggested by increased pulse rates and increased glucagon levels; another possibility is that the rather stressful experimental conditions(catheter insertion and tissue biopsies) have contributed. Nevertheless, any systemic stress response has been similar in both legs, so any observed leg difference must be due to direct local LPS action.

The strength of the methodology is that it excludes day-to-day intrapersonal variability and eliminates the need for an extra control experiment. On the other hand, the AV model has some limitations. One of the most prominent is the large variability in blood flow (39), which introduces a similar variability in calculated AV substrate fluxes. This variability may be further aggravated by the fact that blood flows, for practical reasons, are often measured before or after blood sampling and often cannot be measured simultaneously in the 2 legs. In our particular setup, regional blood flow may also be affected by the femoral artery catheter placements. In our experience, pure AV concentration differences are less prone to type 2 errors, because they clearly reduce the variability related

to limb flow. For these reasons we have primarily given our results as pure AV differences in concentrations or isotopic dilution. In addition, future studies could include assessment of mitochondrial function, nitric oxide levels, and lipid content. It also remains possible that other modes of administration (eg, bolus), other dosages, and other time points of sampling would have yielded other results. Nevertheless, our results are in good accordance with previous studies showing insulin resistance and increased lipolysis after LPS administration.

In summary, constant intraarterial LPS directly inhibits insulin-stimulated glucose uptake and increases palmitate release and isotope dilution in the perfused human leg without detectable effects on amino acid metabolism or lactate and cytokine release, suggesting that insulin resistance may be caused by FFA. These data strongly suggest that the primary metabolic effect of LPS is increased lipolysis and muscle insulin resistance, which together with secondary insulin resistance caused by cytokines and stress hormones may lead to overt glucose intolerance and diabetes.

Acknowledgments

Address all correspondence and requests for reprints to: Ermina Bosnjak, Medical Research Laboratories, Aarhus University Hospital, NBG, Norrebrogade 44, DK-8000 Aarhus C, Denmark. E mail: ermina.bosnjak@ki.au.dk.

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