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Chronic Hepatic Inflammation Induced by Mild Portal Endotoxemia is not Associated with Systemic Insulin Resistance in Rats

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Background: Portal endotoxemia has been speculated to be crucially involved in the pathogenesis of chronic hepatic inflammation which is highly associated with the development of type 2 diabetes mellitus (T2DM). This study examined whether portal endotoxemia was a pathogenic link between chronic subacute hepatic inflammation and systemic insulin resistance. **Methods:** Rats were randomly assigned into four groups: rats with intraportal saline or with three different doses of lipopolysaccharide (LPS) infusion for 4 wks. Pathological changes in the liver were evaluated via histological and biochemical examination. Systemic insulin sensitivity was evaluated by euglycemic hyperinsulinemic clamp by the tracer dilution method. **Results:** Body weight, fasting whole blood glucose, plasma insulin, triglyceride levels, mean arterial blood pressure and heart rate were not significantly changed in the four-week infusion period for all groups. The biochemical indicators of hepatic function such as aspartate aminotransferase, alanine aminotransferase and albumin were not changed during the observed period for all groups. During the euglycemic hyperinsulinemic clamp period, the ratio of glucose infusion rate to insulin level, an index of whole body insulin sensitivity, did not change among groups. Accordingly, insulin-suppressed hepatic glucose production and insulin-stimulated whole body glucose utilization also failed to change in any of the groups at the end of the study. The histopatholgical examination revealed that intraportal low-dose LPS infusion progressively enhanced the inflammatory changes of the liver in experimental rats in a dose-dependent manner. **Conclusion:** The present study suggests that mild portal endotoxemia could induce chronic low-grade hepatic inflammation but does not significantly affect systemic insulin sensitivity in the present experimental condition.

Key words: portal endotoxemia, chronic low-grade hepatic inflammation, insulin resistance, rats

INTRODUCTION

Portal endotoxemia has been proposed to be causally linked with non-alcoholic steatohepatitis (NASH) and also alcoholic liver disease. Accordingly, clinical and experimental studies have implicated that gut-derived endotoxin might play a role in the initiation and aggravation of alcohol-induced liver disease.[1,2] These observations suggest that portal endotoxemia might significantly contribute to the pathogenesis of chronic hepatic inflammation. Furthermore, our recent study demonstrates that chronic low-dose infusion of endotoxin into the portal vein is sufficient to cause liver injury in the absence of steatosis and ethanol intoxication.[3]

On the other hand, clinical studies associated with chronic hepatic inflammation such as alcoholic and non-alcoholic steatohepatitis and chronic viral hepatitis focus in particular on metabolic syndrome (MS)[4,5], and type 2 diabetes mellitus (T2DM).[6,7] Using mice with selectively expressing constitutively active IKK-β in liver, Cai et al showed that the transgenic mice exhibit a type 2 diabetes phenotype, characterized by hyperglycemia, profound hepatic insulin resistance, and moderate systemic insulin resistance.[8] However, the pathogenic link between chronic hepatic inflammation induced by portal endotoxemia and insulin resistance remains elusive.

In addition, we conducted a study in a non-obese ro-

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dent model combined with a novel method of intraportal low-dose lipopolysacchoride (LPS) infusion. Our previous study showed that the appropriate intraportal LPS infusion rate produces a low-grade hepatic inflammation but does not significantly elevate circulating endotoxin level.[9] The current study was intended to examine whether the pathological elevation in portal endotoxin level could induce chronic hepatic inflammation and also affect peripheral insulin sensitivity.

METHODS

Animals and surgical procedures

Male Sprague-Dawley (SD) rats (5-6 weeks old) were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). The rats were housed in an Association of Assessment and Accrediation of Laboratory Animal Care (AAALAC)-certified animal center. All animals were handled and housed according to the guidelines and manual of the institutional animal care and use committee of this institute.

Rats weighing 250-300 g were randomly assigned into two groups (n=8 per group): rats with intraportal vehicle infusion (CPV), and rats with three different-doses (0.42, 0.84 and 1.68 ng/kg/min) of intraportal LPS (lipopolysaccharide *Escherichia coli* serotype 055:B5, Sigma Co. St Louis, Missouri, USA) infusion (C_{PLPSx1}, C_{PLPSx2}, C_{PLPSx4}). A laparotomy was performed under anesthesia (sodium pentobarbital 25 mg/kg using intraperitoneal injection) in rats with intraportal infusion. A catheter (PE-5 tubing, 0.008 inch inner diameter x 0.020 inch outer diameter, SCI micro medical grade polyethylene, Scientific Commodities Inc., Lake Havasu City, AZ, USA) was inserted into the distal end of a colic vein and the tip of the colic catheter was placed about 3 mm distal to the point at which the catheterized vein enters the portal vein. After insertion, the catheters were filled with LPS-containing saline or saline alone. Their free ends were connected to osmotic minipumps (model 2004, Alzet corporation, Cupertino, CA, USA) filled with LPS or saline. The osmotic minipump was then placed in a subcutaneous pocket to allow complete closure of the incision. The selected dose of LPS infusion was based on evaluating its effects on the mortality rate during a 4-wk observation period and on systemic endotoxin levels as shown in our previous study.[3]

At the end of week 4, under anesthesia vascular catheters (micro-renathane implantation tubing, model number: MRE 040, 0.04 inch outer diameter x 0.25 inch inner diameter and model number: MRE 033, 0.33 inch outer diameter x 0.14 inch inner diameter) were placed in the left femoral artery and right femoral vein, respectively. Their proximal ends were placed in subcutaneous pockets under the scapular area. After recovery for 3-4 days, the rats were studied only if they had restored preoperation body weight and normal stools. On the morning following the acute study, the proximal ends of the catheters were exterized and cleared. Thus, intravenous & intra-arterial accesses were established.

Euglycemic Hyperinsulinemic Clamp (EHC) Experiments

The experiment was started in rats after an overnight fast. The experiment consisted of a 30-min basal followed by a 120-min EHC period. Intravenous infusion of [3-^{3}H]glucose (PerkinElmer, Boston, MA, prime 4.8 μCi, followed by 0.08 and 0.16 μCi/min in basal and EHC periods) was begun with a 40-min equilibration period before the basal period and continued throughout the experiment. During the EHC period, variable doses of glucose and constant insulin infusion rate (8 mU/kg/min, porcine regular insulin, Actrapid, Novo Nordisk A/S, Denmark) were given to create euglycemic hyperinsulinemia and to evaluate insulin-dependent glucose metabolism in all groups. One hour after starting the EHC period, three blood samples (0.4 ml each) at 30 min interval (time 60, 90, 120 min) were collected from the femoral artery and the average of the measurements from these blood samples represented the mean value of the clamp period. The total blood volume withdrawn over the experimental period was controlled at an amount of no more than 3 ml/rat and an equal volume of saline was simultaneously administered via the infusion line.

Blood pressure measurement

A mean arterial blood pressure (MAP) measurement was performed in conscious rats at the end of wks 0, 4, 8 and 12 by an indirect tail-cuff method (volume-oscillometric method) using a fully automatic blood pressure monitoring system (UR-5000, UEDA, Japan) as described before.[10]

Hematoxylin/eosin (H/E) stain

In the second set of rats (n=5-6 per group), the same grouping was used as in the above experiment. The perfused liver was then fixed in 5% zinc-formalin solution and embedded in paraffin. Thereafter, these tissue slices were prepared for staining with H/E to evaluate pathological changes. Each liver tissue specimen was scored according to the criteria proposed by Lee.[11] The number

Table 1 BW, MAP, heart rate, whole blood glucose, plasma insulin and triglyceride concentrations before and after 2 and 4 weeks of osmotic minipump implication

	Pump implantation	BW g	MAP mmHg	HR bpm	Glu mmol/L	Ins pg/ml	TG mg/dl
Pv	0	273±4	107±5	360±11	4.1±0.4	0.5±0.1	69.8±7.7
	2	323±11	111±3	348±5	3.6±0.3		83.3±11.9
	4	407±15	110±3	337±7	4.0±0.2	0.9±0.1	78.8±15.9
P_{LPSx1}	0	270±5	101±4	351±16	3.9±0.2	0.7±0.2	108.9±9.6
	2	315±16	113±2	380±16	3.8±0.1		84.6±9.4
	4	418±8	111±3	338±18	4.0±0.2	0.8±0.2	89.0±11.4
P_{LPSx2}	0	289±9	105±3	380±9	4.0±0.1	0.7±0.2	55.6± 8.0
	2	357±8	107±3	372±8	4.3±0.3		67.7±13.0
	4	411±8	113±3	336±10	3.8±0.2	0.9±0.2	70.7±10.3
P_{LPSx4}	0	265±15	105±3	377±20	3.9±0.2	1.0±0.2	85.8±16.1
	2	328±14	107±3	382±21	5.3±0.7*		86.8±11.2
	4	376±9*	105±3	330±16	3.8±0.4	1.1±0.2	81.8±20.5

BW, body weight; MAP, mean arterial blood pressure; HR, heart rate; Glu, whole blood glucose; TG, plasma triglyceride. CONT, control rats; Pv, rats with intraportal vehicle infusion; P_{LPSx1}, rats with intraportal LPS infusion, 0.042 ng/kg/min; P_{LPSx2}, rats with intraportal LPS infusion, 0.084 ng/kg/min; P_{LPSx4}, rats with intraportal LPS infusion, 0.168 ng/kg/min. *P<0.05 vs. Pv in the corresponding period.

of foci per low-power field defined the degree of inflammation (absent, 0; 1 focus, 1+; 2 or more foci, 2+; bridging or confluent, 3+).

Biochemical analysis

After overnight fasting, arterial blood was collected. The whole blood glucose level was assayed by the glucose oxidase method. Plasma insulin level was measured by the commercial rat ELISA kits provided by Mercodia AB (Uppsala, Sweden). Liver homogenates were prepared and analyzed by the commercial rat TNFα ELISA kits (R & D systems, Minneapolis, MN). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin and amylase were measured by Randox reagent kits (Randox Laboratories LTD. Antrim, United Kingdom).

Statistical analysis

Statistical analysis was performed according to the repeated measurements of one-way analysis of variance (ANOVA) followed by the Bonferroni test. A possibility of P <0.05 was taken to indicate a significant difference between means. Values are expressed as means±SEM.

RESULTS

Metabolic and hemodynamic parameters

As shown in Table 1, there were no significant differences in MAP, heart rate, fasting plasma glucose, insulin and triglyceride levels during LPS or the vehicle-infused period among experimental groups. Body weight was not significantly changed among groups in the corresponding period except that in 4-wk. The LPS-infused group P_{LPSx4} was slightly lower than PV in the same time period. AST, ALT and albumin were not significantly changed in any of the groups during the study period as shown in Table 2.

Table 2 Plasma aspartate aminotransferase, alanine aminotransferase and albumin concentrations before and after 2 and 4 weeks of minipump implication

	Pump implantation	AST mg/dl	ALT mg/dl	Albumin g/l
Pv	0	96.0±7.3	44.5±8.6	38.5±2.4
	2	81.3±9.1	37.0±4.9	42.8±3.3
	4	77.8±2.1	33.0±4.2	42.5±5.2
P_{LPSx1}	0	97.0±2.8	45.9±2.5	33.1±1.6
	2	99.4±13.7	46.1±5.9	36.0±3.4
	4	107.3±13.7	39.1±4.0	36.4±2.8
P_{LPSx2}	0	90.3±4.5	36.8±4.5	39.8±1.9
	2	79.2±11.4	40.3±4.1	38.6±2.5
	4	88.8±5.2	33.8±2.8	38.5±2.0
P_{LPSx4}	0	118.3±24.7	40.8±2.7	42.0±3
	2	91.5±11.5	46.3±2.7	37.9±1.8
	4	90.8±1.2	42.0±5.0	40.8±2.5

AST, aspartate aminotransferase; ALT, alanine aminotransferase. Pv, rats with portal vehicle infusion; Pv, rats with intraportal vehicle infusion; P_{LPSx1},rats with intraportal LPS infusion, 0.042 ng/kg/min; P_{LPSx2}, rats with intraportal LPS infusion, 0.084 ng/kg/min; P_{LPSx4}, rats with intraportal LPS infusion, 0.168 ng/kg/min.

Table 3 MAP, heart rate, whole blood glucose, plasma insulin concentrations and glucose infusion rate during the EHC study.

		MAP mmHg	HR bpm	Glucose mmol/L	Insulin pg/ml	GIR/Insulin
Pv	Cont	118±5	391±12	4.9±0.2	0.8±0.2	
	EHC	131±3	385±8	4.6±0.2	20.3±2.1	1.65±0.36
P_{LPSx1}	Cont	113±2	358±16	4.2±0.2	0.9±0.1	
	EHC	130±2	364±16	4.5±0.2	18.3±1.6	1.96±0.24
P_{LPSx2}	Cont	116±3	382±11	4.5±0.1	1.1±0.1	
	EHC	127±4	368±9	4.4±0.1	16.5±1.9	1.94±0.19
P_{LPSx4}	Cont	103±4	376±15	4.6±0.3	1.0±0.1	
	EHC	120±4	388±14	4.4±0.3	19.3±2.4	1.62±0.27

MAP, mean arterial blood pressure; HR, heart rate; Glu, whole blood glucose; Insulin, plasma insulin,; GIR, glucose infusion rate. Indicates CONT, control rats; Pv, rats with intraportal vehicle infusion; P_{LPSx1},rats with intraportal LPS infusion, 0.042 ng/kg/min; P_{LPSx2}, rats with intraportal LPS infusion, 0.084 ng/kg/min; P_{LPSx4}, rats with intraportal LPS infusion, 0.168 ng/kg/min.

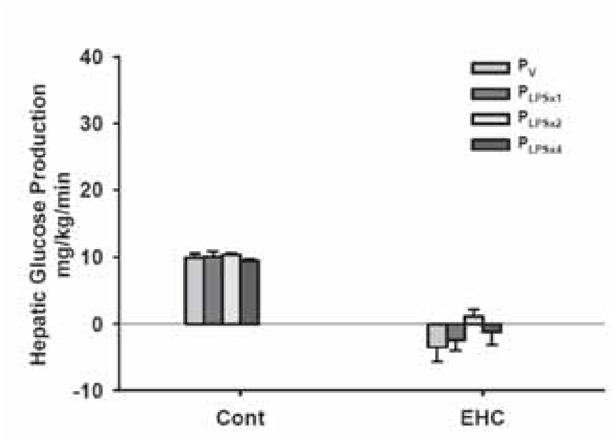

Fig 1 Hepatic glucose production during the euglycemic hyperinsulinemic clamp (EHC) period. Pv, rats with portal vehicle infusion; P_{LPSx1}, rats with intraportal LPS infusion, 0.042 ng/kg/min; P_{LPSx2}, rats with intraportal LPS infusion, 0.084 ng/kg/min; P_{LPSx4}, rats with intraportal LPS infusion, 0.126 ng/kg/min.

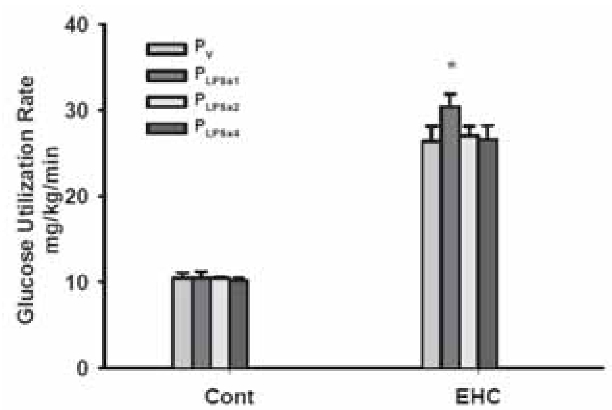

Fig 2 Whole body glucose uptake during the EHC period. Pv, rats with portal vehicle infusion; P_{LPSx1},rats with intraportal LPS infusion, 0.042 ng/kg/min; P_{LPSx2}, rats with intraportal LPS infusion, 0.084 ng/kg/min; P_{LPSx4}, rats with intraportal LPS infusion, 0.126 ng/kg/min. *P<0.05 vs. CONT in the corresponding period.

Euglycemic hyperinsulinemic clamp

As shown in Table 3, there were no significant changes in observed parameters during the basal period. During the EHC period, variable glucose combined with high insulin infusion created similar euglycemia and hyperinsulinemia among all groups. The ratio of GIR to insulin, an index of insulin sensitivity, was not significantly different in any groups.

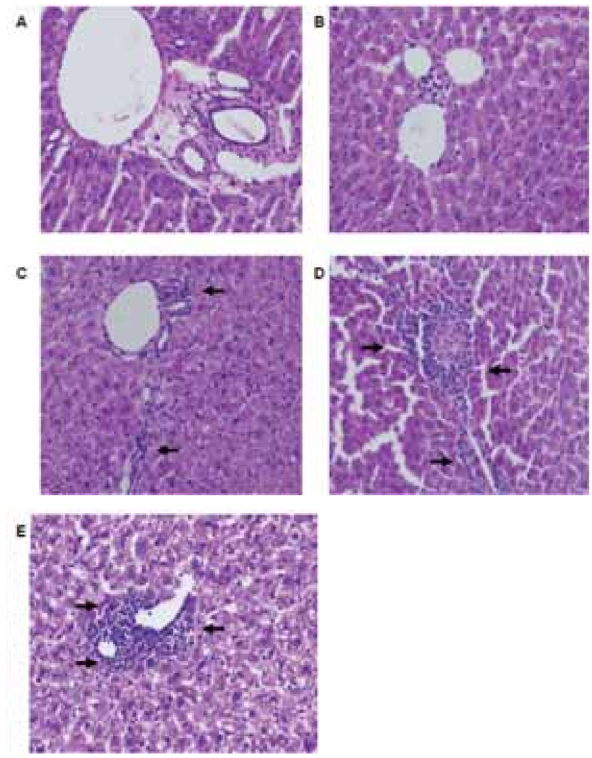

Fig 3. The pathology of the liver in the experimental groups. (A) CONT (B) Pv (C) P_{LPSx1} (D) P_{LPSx2} (E) P_{LPSx4}. Abbreviations have been indicated in Figure 1. Arrows indicate neutrophil infiltration. (H&E stain, x 400）

Figure 1 shows that the hepatic glucose production rate was not significantly changed among any groups during the basal and EHC periods. In addition, the glucose utilization rate was also not significantly altered among groups during the basal and EHC periods as shown in Figure 2.

Histopathologic examination of liver

As shown in Figure 3, the H and E stain showed that the observed neutrophil cell infiltration was primarily confined to areas around the central vein in P_{PLPSx1} and the infiltrated round cells increased progressively in P_{PLPSx2} and P_{PLPSx4} in a dose-dependent manner. Accordingly, hepatic TNFα contents and the degree of inflammation were dose-dependently increased in rats with chronic intraportal LPS infusion, as shown in Figure 4.

DISCUSSION

The present study demonstrated that mild portal endotoxemia created by intraportal low-dose LPS infusion

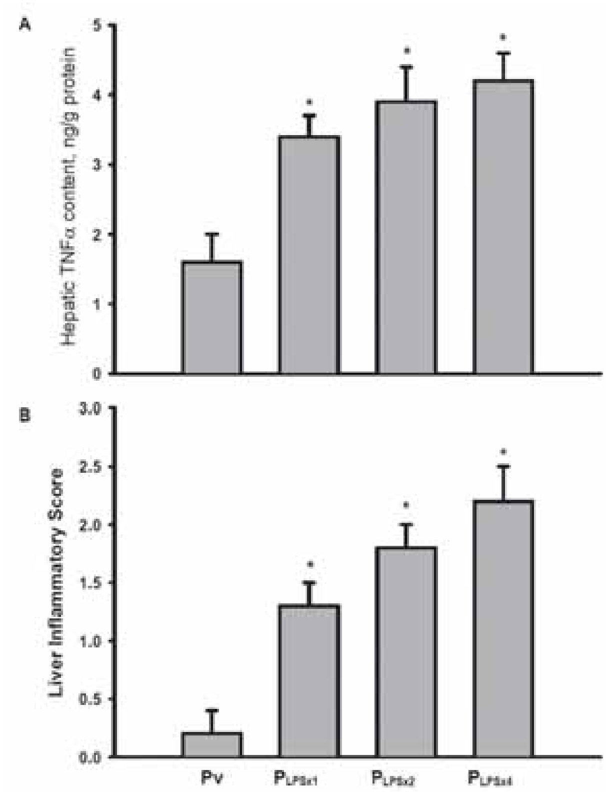

Fig 4 Hepatic TNFα contents (A) and liver inflammatory score (B) in the experimental rats. Abbreviations of groups have been indicated in Figure 1.

is causally linked to subacute hepatic inflammation but does not significantly affect systemic insulin resistance under the present experimental condition.

In our previous study, our data showed that similar dosage ranges of intraportal LPS infusion not only lead to hepatic inflammation but also low-grade pancreatic inflammation and impairment of the first and second-phase insulin secretion in rats with normal livers and fructose-induced fatty liver.[3,9] Taken together with the present results, our observations suggest that chronic low-grade portal endotoximia might play a pivotal role in the pathogenic link between chronic liver diseases and the development of T2DM. Furthermore, this mild portal endotoxemia might deteriorate the progression of T2DM mainly by impairing pancreatic insulin secretion but not by affecting the insulin resistance of the whole body.

A topic that has intrigued relevant investigators is that the failure to perform the normal function of detoxifying endotoxin might initiate or perpetuate liver injury, or lead to systemic effects. However, lack of appropriate animal models limits the progress of relevant investigations. Using this novel chronic portal endotoxemic rodent model, we evaluate the potential link of portal-endotoxemia-induced hepatic inflammation and extrahepatic insulin resistance. Our data show that chronic low-dose LPS infusion did significantly cause hepatic inflammation but did not affect the status of systemic insulin resistance indicated mainly by the results of the euglycemic hyper-insulinemic clamp study (the gold standard method to evaluate insulin sensitivity *in vivo*)[12] as well as fasting glucose and insulin levels. On the other hand, a recent study by Cai et al showed that NF-kB and transcriptional targets are activated in the liver by obesity and a high-fat diet. In addition, mice with selectively expressing constitutively active IKK-β in the liver exhibit a type 2 diabetes phenotype, characterized by hyperglycemia, profound hepatic insulin resistance, and moderate systemic insulin resistance. The discrepancy between the two studies might cause different conclusions. First, the method to induce liver inflammation is different. In our study, we mimic the natural route of intestinal LPS to induce hepatic inflammation. Whereas the study by Cai et al[8] uses selective gene manipulation. Secondly, the species conducted in the studies are different. However, these observations combined with our previous study in this model implicate that both chronic low-dose endotoxemia and hepatic inflammation might be causally linked to the progression of type 2 DM by different underlying mechanisms.

In conclusion, the present results suggest that portal endotoxemia is a significant contributor to hepatic inflammation but does not significantly affect peripheral insulin resistance in the current experimental situation.

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