Effect of Diabetes Mellitus on Endotoxin-Induced Lung Injury

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Objective: To examine the effects of diabetes mellitus on lipopolysaccharide (LPS)–induced pulmonary edema and alveolar neutrophil recruitment and activation.

Hypothesis: Zucker diabetic fatty rats are resistant to the effects of intratracheal LPS on the extravasation of plasma proteins into the lungs.

Design: Zucker diabetic fatty (ZDF) rats (genotype fa/fa) were used as a model of diabetes mellitus, while their normoglycemic heterozygous littermates served as controls. Lipopolysaccharide (Escherichia coli 0111: B4; 100-200 µg) or vehicle (0.25 mL of isotonic sodium chloride solution) was instilled into the airways of ZDF and control rats. Four hours later, pulmonary microvascular dysfunction was assessed by measuring the extravasation of Evans blue dye into the lung. Lipopolysaccharide-induced neutrophil recruitment was assessed by counting the number of neutrophils within the bronchoalveolar lavage fluid and measuring their expression of CD11b/CD18 by fluorescence-activated cell analysis sorting.

Results: The LPS (200 µg) induced a 32% increase in Evans blue dye extravasation into the lungs of controls (P = .008) but had no such effect in diabetic animals. Pulmonary extravasation of Evans blue dye in controls was greater than that of ZDF rats both at baseline (P = .002) and in response to 200 µg of LPS (P<.001). The LPS up-regulated neutrophil CD11b/CD18 expression in diabetic and nondiabetic groups and induced a greater than 50-fold increase in the number of neutrophils within the airways of both control and diabetic groups (P<.001).

Conclusion: Despite the recruitment of a large number of neutrophils into the lung, the LPS-induced change in pulmonary microvascular permeability in diabetic animals is substantially less than that of nondiabetic controls.

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The pulmonary microvasculature is an important target of the systemic inflammatory response in critically ill patients. Increases in microvascular permeability and alterations in local perfusion result in hypoxemia, often necessitating mechanical ventilation and aggressive pulmonary support. Nearly all of the experimental observations regarding the lung’s response to local and systemic inflammatory mediators have occurred in young, otherwise healthy animal models. Although this strategy has allowed a more focused examination of the physiological and immunological features of inflammation, it has ignored the fact that many critically ill patients have coexisting chronic illnesses that undoubtedly influence the patient’s physiological response to inflammatory mediators.

Since the microvasculature is an important target of diabetes mellitus, we postulated that the pulmonary microvascular responsiveness of diabetic animals to systemic inflammation would be substantially different from that of nondiabetic animals. In preliminary studies that used an isolated, perfused lung model, we found the pulmonary microvasculature of diabetic animals to be significantly less responsive to thromboxane-induced increases in permeability than that of normal animals (unpublished data, 1999). The purpose of this study was to extend our observations regarding the effect of diabetes mellitus on pulmonary microvascular function to a more clinically relevant model of acute inflammatory lung injury. Specifically, this study examines the hypothesis that Zucker diabetic fatty (ZDF) rats are resistant to the effects of intratracheal endotoxin (lipopolysaccharide [LPS]) on the extravasation of plasma proteins into the lungs.

MEASUREMENT OF LPS-INDUCED ACUTE LUNG INJURY

Extravasation of EBD into the lungs of the ZDF and control animals exposed to 0, 100,
MATERIALS AND METHODS

ANIMAL MODEL OF TYPE 2 DIABETES MELLITUS

Male ZDF rats (21-26 weeks of age; 400-600 g) were obtained from a colony at the Dallas Veterans Affairs Medical Center, Dallas, Tex. These animals possess a mutation of the leptin receptor that is phenotypically expressed as obesity, hypertriglyceridemia, and hyperglycemia by 7 to 9 weeks of age; as such, they have been commonly used as an animal model of type 2 diabetes mellitus. Controls consisted of age-matched, lean, normoglycemic male heterozygotic rats.

These experiments were reviewed and approved by the Subcommittee on the Care and Use of Animals in Research at the University of Texas Southwestern Medical School and the Dallas Veterans Affairs Medical Center.

EXPERIMENTAL PROTOCOL

The ZDF and control animals were anesthetized with pentobarbital sodium (40 mg/kg of lean body weight, intraperitoneally) and the trachea was exposed through a small cervical incision. Escherichia coli LPS (100 or 200 µg in 250 µL of sterile isotonic sodium chloride solution; E coli serotype 0111:B4; Sigma-Aldrich Corp, St Louis, Mo) or an equal volume of sterile isotonic sodium chloride solution was injected rapidly into the trachea during inspiration by means of a 27-gauge needle. The wound was then closed and the animals were allowed to recover. This method induces an acute lung injury manifested by increased epithelial and microvascular permeability, neutrophil sequestration, and interstitial edema. Pulmonary microvascular leak (as assessed by Evans blue dye [EBD] extravasation) and neutrophil activation (CD11b/CD18 expression) and sequestration within the lung were assessed 4 hours after the instillation of LPS into the airways. This time point was chosen on the basis of preliminary experiments demonstrating maximal extravasation of plasma proteins into the lung at 4 to 8 hours after exposure to endotoxin. By 24 to 48 hours after injury, the microvascular leak is significantly attenuated, with concentrations of dye within the lungs being no different from that in controls (unpublished data, 1998).

MEASUREMENT OF ACUTE LUNG INJURY

Evans blue dye has been used to quantitate protein extravasation during acute inflammatory events. Previous studies have demonstrated that, after intravenous infusion, more than 99% of the dye exists bound to plasma proteins. This method has been shown to compare favorably with the use of radiolabeled albumin as a marker of plasma protein extravasation and, hence, microvascular “leakiness” during acute inflammatory events.

Three hours after the administration of LPS or isotonic sodium chloride solution, EBD (20 mg/kg) was infused into the femoral vein of the ZDF and control animals (n = 5-8 per group). Sixty minutes later, the heart and lungs were excised and the pulmonary vasculature was flushed free of blood by gently infusing sterile saline into the beating right ventricle. The left lower lobe of the lung was then excised, weighed, and placed in 5 mL of formamide. The tissue was homogenized and incubated in formamide for 18 hours at 37°C. The tissue suspension was then centrifuged at 12 000 rpm for 20 minutes, after which the supernatant was collected and its optical density determined spectrophotometrically (OD480). The amount of dye contained within the sample was extrapolated by means of a standard curve and expressed as milligrams of dye per gram of wet lung weight.

MEASUREMENT OF NEUTROPHIL ACTIVATION AND RECRUITMENT INTO THE LUNG

Quantitation of Neutrophils Within the Airways

In a separate set of experiments, bronchoalveolar lavage (BAL) was performed 4 hours after exposure to LPS (200 µg) or an equivalent volume of sterile isotonic sodium chloride solution. In these experiments, 7 mL of sterile isotonic sodium chloride solution was instilled into the right lung 3 times. The lavage fluid was collected and pooled, and the total cell count was obtained with an automated cell counter. The number of neutrophils present within the lavage fluid was determined by light microscopy after Wright staining.

Quantitation of Neutrophil CD11b/CD18 Expression

An aliquot of BAL fluid was collected to quantitate the expression of the adhesion glycoproteins CD11b and CD18 on neutrophils. In this experiment, neutrophils isolated from the BAL fluid were stained with fluorescein isothiocyanate-conjugated mouse anti–rat monoclonal antibodies against CD11b and CD18 (Pharmigen Inc, San Diego, Calif). An isotypic IgG was used as a control. Cell staining was quantitated by fluorescence-activated cell analysis sorting (FACSTAR; Beckton-Dickinson Co, San Jose, Calif) and expressed as the percentage of cells stained by the fluorescein isothiocyanate–labeled antibodies. A minimum of 10 000 cells were examined per sample.

STATISTICAL ANALYSIS

All data are expressed as mean ± SEM and analyzed by analysis of variance with a Fisher post hoc test. A P value of less than .05 was considered a statistically significant difference between groups.

or 200 µg of LPS is shown in Figure 1. In the absence of LPS, the amount of EBD within the lungs of diabetic animals was about 33% less than that of nondiabetic controls (P = .002). The injection of LPS into the trachea of the control animals resulted in a dose-dependent increase in the extravasation of EBD into the lungs, with a 32% increase for the 200-µg dose (P = .008). In contrast, the injection of even 200 µg of LPS into the airways of the ZDF rats had no significant effect on the concentration of EBD within the lungs when compared with that of ZDF animals exposed to isotonic sodium chloride solution alone. The concentration of EBD within the lungs of control animals exposed to 200 µg of LPS was more than twice that of similarly treated ZDF animals (P < .001).
MEASUREMENT OF LPS-INDUCED NEUTROPHIL SEQUESTRATION

The effect of the ZDF phenotype on LPS-induced neutrophil sequestration within the airways is shown in Figure 2. There were minimal numbers of neutrophils within the airways of either the ZDF or control animals exposed to isotonic sodium chloride solution alone, whereas the injection of 200 µg of LPS into the airways resulted in a large influx of neutrophils. There was no difference in the numbers of neutrophils within the BAL fluid of control or ZDF animals exposed to either isotonic sodium chloride solution or LPS.

MEASUREMENT OF LPS-INDUCED NEUTROPHIL CD11b/CD18 EXPRESSION

The effect of the ZDF phenotype on LPS-induced neutrophil CD11b expression is shown in Figure 3. In the absence of LPS stimulation, about 10% of the neutrophils isolated from the airways expressed CD11b. Four hours after exposure to intratracheal LPS, about 70% of the neutrophils isolated from BAL fluid expressed CD11b (P < .001 vs neutrophils isolated from animals exposed to isotonic sodium chloride solution vehicle alone). There was no difference in the expression of CD11b in response to either intratracheal isotonic sodium chloride solution or LPS on comparison of the ZDF and control groups. Measurements of neutrophil CD18 expression mirrored that of CD11b (data not shown).

The frequency of type 2 diabetes mellitus in the population in general, and in hospitalized patients in particular, makes it imperative that clinicians have a better understanding of the effects of diabetes on the local and systemic inflammatory response to injury, proinflammatory mediators, and infection. The purpose of this study was to examine the effect of diabetes mellitus on the pulmonary microvascular response to intratracheal endotoxin exposure. The results of this study suggest that the pulmonary microvasculature of diabetic animals is significantly less permeable to plasma proteins both at baseline and in response to intratracheal LPS than that of normoglycemic controls. This difference in microvascular protein extravasation occurs despite similar degrees of neutrophil adhesion molecule expression and neutrophil sequestration within the lungs of the diabetic and nondiabetic groups.

The intratracheal instillation of LPS into rats is a commonly used model of acute inflammatory lung injury, and, as such, it has been used to investigate the pathophysiological characteristics of acute respiratory distress syndrome. The experimental model is associated with hypoxemia, impaired surfactant production, neutrophil sequestration within the lungs, and pulmonary edema with parenchymal consolidation. The increased microvascular and epithelial permeability associated with

**Figure 1.** The concentration of Evans blue dye (EBD) within the lungs of obese diabetic rats (black bars; n = 4-8 per group) or lean normoglycemic heterozygotic rats (gray bars; n = 4-8 per group) 4 hours after exposure to vehicle (sterile isotonic sodium chloride solution) or lipopolysaccharide (LPS) administered intratracheally. Asterisk indicates P < .005 vs similarly treated heterozygotes; dagger, P = .008 vs isotonic sodium chloride solution–treated heterozygotes.

**Figure 2.** The neutrophil count within the airways of obese diabetic rats or lean normoglycemic heterozygotic rats exposed to sterile isotonic sodium chloride solution (gray bars; n = 2 per group) or 200 µg of lipopolysaccharide (black bars; n = 3 per group). The neutrophils were recovered by bronchoalveolar lavage 4 hours after exposure to isotonic sodium chloride solution or lipopolysaccharide. Asterisk indicates P < .001 vs isotonic sodium chloride solution–treated animals of a similar phenotype.

**Figure 3.** The effect of intratracheal lipopolysaccharide (200 µg) on the expression of the neutrophil adhesion glycoprotein CD11b. The gray bars represent animals exposed to sterile isotonic sodium chloride solution (n = 3 per group). The black bars represent animals exposed to 200 µg of lipopolysaccharide (n = 3 per group). Asterisk indicates P < .001 vs isotonic sodium chloride solution–treated animals of a similar phenotype. PMN indicates polymorphonuclear neutrophil.
Diabetes mellitus is one of the most common chronic diseases in the United States, being present in nearly 20% of hospitalized patients. Despite this nearly unparalleled prevalence, little is known regarding the effect of diabetes on the pulmonary microvascular response to injury. The present study utilizes a commonly employed animal model of type 2 diabetes mellitus to examine its effect on the pulmonary microvascular response to endotoxin. These experiments suggest that the pulmonary microvasculature of diabetic animals is significantly less responsive to intratracheal endotoxin than is that of nondiabetic controls, despite similar degrees of neutrophil activation and sequestration within the airways. A similar phenomenon in patients with diabetes mellitus may impair the ability of diabetic patients to clear infectious challenges from the lungs. Conversely, this blunted microvascular responsiveness may limit the pulmonary edema that characterizes many systemic inflammatory states.

In summary, these data suggest that the pulmonary microvascular response to intratracheal endotoxin is severely blunted in diabetic rats when compared with that of normoglycemic controls. This occurs despite similar degrees of neutrophil CD11b/CD18 expression and pulmonary neutrophil sequestration within the lungs. These studies support the contention that chronic illnesses such as diabetes mellitus may significantly alter the responsiveness of an organ to acute inflammatory stimuli. This assumes particular importance given the frequent incidence of chronic illnesses among critically ill patients.

REFERENCES


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With regard to a more relevant model, such as bacteria injection, we do have some preliminary studies where we have injected live organisms down the trachea of these animals and have just done some survival studies to see how long they last, and preliminarily, it looks like the diabetic animals do not survive as well. After about 4 or 5 days, most of those animals have died, whereas relatively few of the nondiabetic animals are killed by that.

Ronald V. Maier, MD, Seattle, Wash: If I understand you correctly from your preliminary data where you used basically a hydrostatic force, mechanical force, and showed decreased leak, and now in this series where you show that you do get neutrophils in the alveolar space, and from pathology it looks like they are activated and inflamed and in the interstitium and you have interstitial fluid but less leak through both mechanisms, I wonder if the explanation is as you mentioned, glycosylation causes lots of cross-linking of proteins, and whether you are just having basically a glue-like mechanism of your basement membrane so that you physically cannot get fluid across into the alveolar space.

And I wonder if you have any evidence or data to support that. These animals are young enough that they probably wouldn't show structural changes on morphology as far as thickening of the basement membrane, but from a functional standpoint as far as permeability, they may just have glycosylated cross-linking that prevents permeability or the increase with thromboxane or give them endotoxin.

Dr Wright: Right. We haven't done any experiments as of yet looking specifically at the cross-linking, although those are in our plans. With aminoguanidine, you can prevent that advanced glycosylation cross-linking of those products, but then you run into problems with nitric oxide, so you don't really know what is causing your injury or preventing it.

In addition, it appears to be very organ-specific, the changes in permeability. Increased glycosylation products and alterations in heparan sulfate, for instance, in basement membranes have been measured in different organs, say, in the kidney, where permeability is increased, or in the lung, where we are showing that it's decreased and other people have also shown it to be decreased. So it is very interesting how it is organ-specific as well as whatever mechanism is involved.

E. Patchen Dellinger, MD, Seattle: Are there any data suggesting that the incidence of ARDS in diabetic patients is different after trauma or in association with SIRS [systemic inflammatory response syndrome], or are there any data regarding the relative incidence at all?

Dr Wright: That is a very good question, and I have looked long and hard for that. I have not been able to find any good studies showing those differences. Talking to some of the pulmonologists, anecdotal cases, they will say that may be the case, that diabetics get less ARDS, but I don't know of any studies that have shown that.

Dr Dellinger: Those SIS [Surgical Infection Society] members with trauma registries might think of searching their database to look for that.

Edwin A. Deitch, MD, Newark, NJ: Permeability is a surrogate for dysfunction. Do you have any O₂ levels, PaO₂ levels, or any other functional assays to say that the lungs of these diabetic animals work better, oxygenate better, ventilate better?

Dr Wright: No, we haven't measured oxygenation or blood arterial oxygen levels.

Cathy L. White Owen, MD, Cleveland, Ohio: Since you are using an isolated perfusion model, is there any possibility that you would consider looking at a cell-free system to try and sort out whether this is a PMN [neutrophil]–related issue vs a lung-related issue, and have you considered doing studies where you used diabetic lungs with normal neutrophils and normal lungs with diabetic neutrophils?

Dr Wright: No, we have not done that.