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PROTECTIVE EFFECT OF A RECOMBINANT FRAGMENT OF BACTERICIDAL/PERMEABILITY INCREASING PROTEIN AGAINST CARBOHYDRATE DYSHOMEOSTASIS AND TUMOR NECROSIS FACTOR-α ELEVATION IN RAT ENDOTOXEMIA

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Abstract—Endotoxin (lipopolysaccharide, LPS), a component of the gram-negative bacterial cell wall, induces carbohydrate dyshomeostasis and the release of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) when administered to experimental animals. Bactericidal/permeability increasing protein (BPI), a cationic protein found in human neutrophil granules, binds with high affinity to LPS and is capable of neutralizing its biological activity. The present study was designed to determine if a recombinant N-terminal fragment of BPI, $rBPI_{23}$, attenuates LPS-induced alterations in serum glucose, lactate, and TNF-α in rats. In anesthetized animals challenged with a 30 min infusion of *Escherichia coli* O111:B4 LPS (0.25 mg/kg), there was an early transient increase in serum levels of glucose followed by a drop to 60% of those found in saline control rats. A prolonged elevation in serum levels of lactate and a transient, but marked, elevation of TNF-α were also observed following LPS infusion. These LPS-induced changes were inhibited significantly by simultaneous infusion of $rBPI_{23}$. Different dose–response profiles of $rBPI_{23}$ on LPS-induced alterations in glucose, lactate and TNF-α were observed. When $rBPI_{23}$ was infused 30 min after the initiation of LPS infusion, it significantly inhibited the alterations in glucose and lactate, but not TNF-α. The rise in TNF-α was reduced significantly with a 15 min delayed infusion of $rBPI_{23}$. A control protein failed to alter any responses to LPS. The results indicate that $rBPI_{23}$ can provide significant protection against the metabolic disturbances and TNF-α release associated with endotoxemia. In addition, the results suggest that LPS-induced metabolic alterations in glucose and lactate are at least partially independent of TNF-α release.

Key words: lipopolysaccharide/endotoxin; BPI; glucose; lactate; cytokines; sepsis/septic shock

Derangements in metabolic homeostasis are prominent components in the pathophysiological response to endotoxemia [1, 2]. Carbohydrate metabolism, in particular, manifests marked alterations, as evidenced by an early transient hyperglycemia and a progressive hypoglycemia [3, 4]. The glucose dyshomeostasis is correlated with the pathogenesis of shock [2], the deterioration of cardiovascular function [5–8], and mortality [8]. Associated with the alterations in glucose is an elevation of blood lactate, a product of anaerobic glycolysis. The concentration of blood lactate has been shown to be a valuable aid in the assessment of the severity, prognosis and response to therapy in shock patients [9–11]. Among the potential mediators of LPS†-induced metabolic alterations are proinflammatory cytokines, particularly TNF-α. For instance, TNF-α administration induces changes in glucose and lactate levels that are similar to those that occur following LPS challenge [12–14]. However, the involvement of a mediator(s) other than TNF-α in the LPS-induced metabolic disturbances remains unclear [14–17].

BPI is a 55-kDa protein found in human neutrophil granules [18, 19]. It binds to a broad spectrum of LPS and has been shown to neutralize the effects of LPS *in vitro* [20–22]. The N-terminal fragment of natural BPI has been shown to exhibit all the biological activities of the holoprotein [23], and was recently cloned and expressed [21, 24]. The recombinant fragment, referred to as $rBPI_{23}$, inhibited LPS- and *Escherichia coli*-induced cytokine release in human whole blood [22, 24], and prevented cytokine release and subsequent death in an actinomycin D-sensitized mouse model of lethal endotoxemia [25].

We also demonstrated recently that $rBPI_{23}$ prevented hyperdynamic responses to infusion of a nonlethal dose of endotoxin in rats [26]. The present study was designed to determine if $rBPI_{23}$ prevents carbohydrate dyshomeostasis and TNF-α elevation induced by a low, nonlethal, infused dose of LPS in rats.

MATERIALS AND METHODS

Reagents. LPS (*E. coli* O111:B4) was obtained

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† Abbreviations: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; and BPI, bactericidal/permeability increasing protein.

from the Sigma Chemical Co. (St. Louis, MO). LPS solution was prepared in pyrogen-free phosphate-buffered saline (GIBCO Laboratories, Grand Island, NY). $rBPI_{23}$ corresponding to the first 199 amino acids of human BPI [27], was cloned, expressed in CHO-K1 cells, and purified from culture medium by cation exchange chromatography as previously described [21]. Thaumatin, isolated from the African plant *Thaumatococcus danielli*, was used as a control protein because it has a similar molecular weight and isoelectric point as $rBPI_{23}$ and does not bind to LPS *in vitro* [21].

Animal preparations and surgery. Male Sprague–Dawley rats (Simonsen Laboratories, Gilroy, CA) weighing between 250 and 350 g were used in all experiments. Animals were housed in conventional cages in the climate- and light/dark cycle-controlled environment of the animal facility of XOMA and received standard laboratory chow and water *ad lib.* The animals were fasted by removing food, but not water, for 24 hr before the experiments examining glucose and lactate levels. Each rat was anesthetized with an intramuscular injection of a mixture of 80 mg/kg ketamine and 4 mg/kg xylazine. Anesthesia was maintained by i.v. infusion through a femoral vein at a dose of 17.8 mg/kg/hr for ketamine and 0.89 mg/kg/hr for xylazine. A silastic catheter was implanted in a femoral vein and connected to an infusion pump to infuse LPS or to maintain anesthesia. Another silastic catheter was implanted in the right jugular vein for infusion of $rBPI_{23}$, control protein, or vehicle.

Experimental protocol. LPS was infused into the femoral vein over 30 min at a dose of 0.25 mg/kg in 1 mL, as previously described [26]. Simultaneously or up to 30 min later, $rBPI_{23}$, control protein, or vehicle was infused into the jugular vein. Saline control animals (unchallenged controls) received an equal volume of saline and vehicle.

Glucose and lactate assays. Blood samples were obtained via the femoral vein catheter every 30 min for the first 2 hr and every hr for the remainder of the 5 hr. The blood samples were placed in microtubes, allowed to clot, and centrifuged to obtain serum. Glucose and lactate were determined using a Glucose/L-Lactate Analyzer (2300 STAT, YSI, Yellow Springs, OH). Lactate levels were not analyzed in control protein-treated rats because control protein interfered with lactate measurements.

TNF-α assay. Serum was collected from separate groups of animals and stored at −70° until it was assayed for levels of TNF-α. Serum levels of TNF-α were determined using an L929 fibroblastoid cell cytolytic assay as described [25]. Recombinant mouse TNF-α (Genzyme Corp., Cambridge, MA) was used to generate a standard curve. The specific activity of the TNF-α standard was approximately 500 U/ng, where one unit is defined as the quantity of TNF-α required to lyse 50% of L929 cells.

Statistical analysis. Data are expressed as means ± SEM. Data were analyzed using repeated measures of variance with Fisher's least-significant-difference test applied where appropriate. Statistical significance was accepted at $P < 0.05$.

RESULTS

Effect of $rBPI_{23}$ on LPS-induced metabolic alterations in glucose. Animals infused with 0.25 mg/kg LPS manifested a two-phase response in serum glucose consisting of an early transient increase followed by a progressive drop to 60% of that found in saline control rats at the end of the experiment (Fig. 1A). In contrast, animals receiving simultaneous infusions of LPS and $rBPI_{23}$ (5 mg/kg) exhibited glucose levels that did not differ from the saline control animals at any time point. Infusion of the control protein did not inhibit the LPS-induced

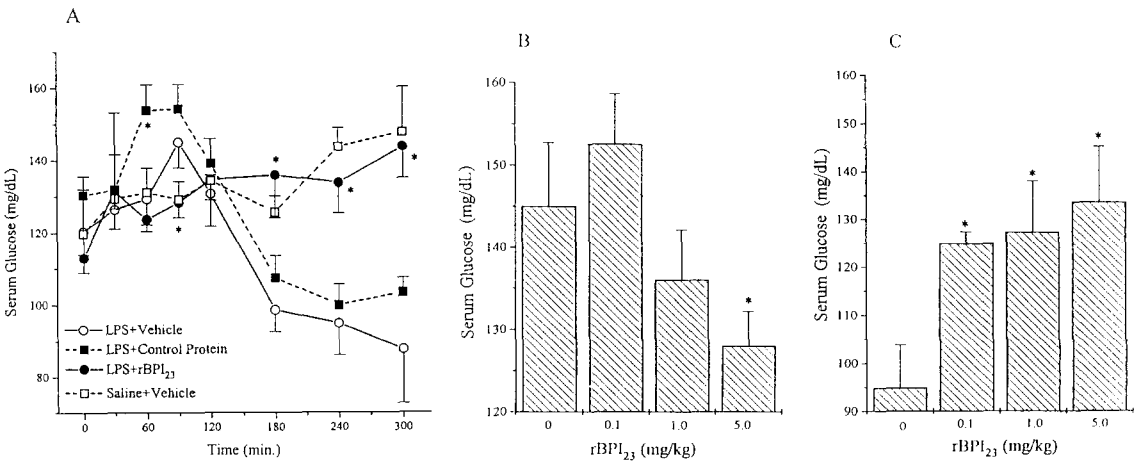

Fig. 1. (A) Effect of $rBPI_{23}$ on LPS-induced alterations in glucose. Rats (8/group) were challenged with 0.25 mg/kg LPS over 30 min and simultaneously infused with $rBPI_{23}$ (5 mg/kg), vehicle, or a control protein. Key: (*) $P < 0.05$ compared with LPS + vehicle. (B and C) Dose-related effect of $rBPI_{23}$ on early and late changes in serum glucose levels. Serum levels of glucose were compared among different $rBPI_{23}$ dose groups (6–8/group) at 90 min (B) and 240 min (C) after the initiation of LPS infusion. Key: (*) $P < 0.05$ compared with LPS + vehicle (0 mg/kg $rBPI_{23}$). All values are means ± SEM.

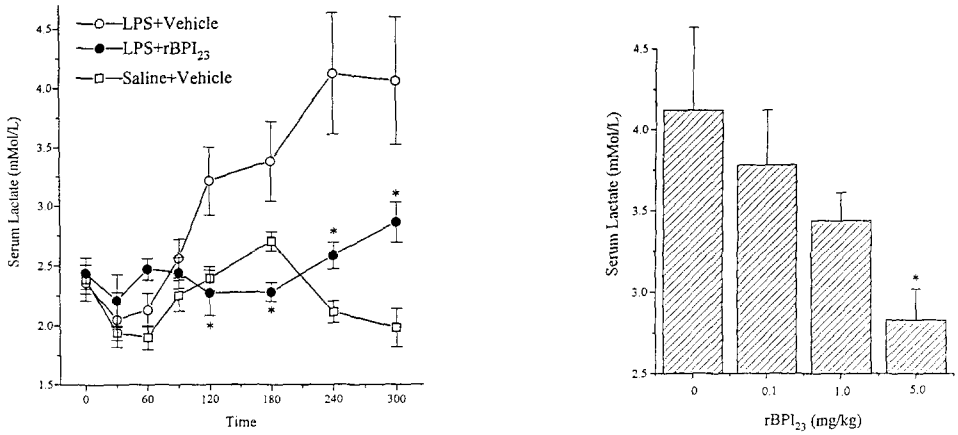

Fig. 2. (Left panel) Effect of rBPI$_{23}$ on LPS-induced hyperlactatemia. Rats (8/group) were challenged with 0.25 mg/kg LPS over 30 min and simultaneously infused with rBPI$_{23}$ (5 mg/kg), vehicle, or a control protein. Key: (*) $P < 0.05$ compared with LPS + vehicle. (Right panel) Dose-related effect of rBPI$_{23}$ on serum lactate. Serum levels of lactate were compared among different rBPI$_{23}$ dose groups at 240 min after initiation of LPS infusion. Key: (*) $P < 0.05$ compared with LPS + vehicle (0 mg/kg rBPI$_{23}$). All values are means ± SEM.

alterations. Dose-related effects of rBPI$_{23}$ on serum glucose levels are shown in Fig. 1, B and C. A minimum of 5 mg/kg rBPI$_{23}$ was required to prevent the early transient hyperglycemia (Fig. 1B), whereas a 50-fold lower dose (0.1 mg/kg) was sufficient to inhibit the later decrease in glucose (Fig. 1C).

Effect of rBPI$_{23}$ on LPS-induced hyperlactatemia. Serum lactate levels started to increase 120 min after the initiation of LPS and vehicle infusions, and reached a peak at 240 min (Fig. 2, left panel). In animals receiving simultaneous infusion of LPS and rBPI$_{23}$ (5 mg/kg), the lactate levels were significantly lower after 120 min, although higher than the saline control group at 240 and 300 min. There appeared to be a dose-dependent inhibition by rBPI$_{23}$ (Fig. 2, right panel). However, only the 5 mg/kg dose provided statistically significant inhibition.

Effect of rBPI$_{23}$ on LPS-induced TNF-α elevation. TNF-α was elevated in the sera of rats following simultaneous infusion of LPS and vehicle (Fig. 3, left panel). The levels peaked at 90 min after the initiation of LPS infusion, and declined thereafter. Simultaneous infusion of 3 mg/kg rBPI$_{23}$ significantly inhibited the LPS-induced elevation of TNF-α. In

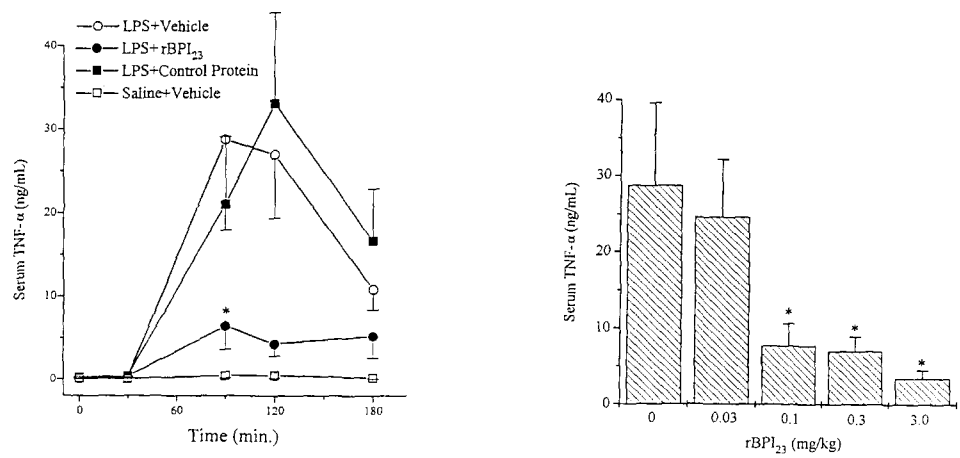

Fig. 3. (Left panel) Effect of rBPI$_{23}$ on LPS-induced TNF-α elevation. Rats (8/group) were challenged with 0.25 mg/kg LPS over 30 min and simultaneously infused with rBPI$_{23}$ (3 mg/kg), vehicle or a control protein. Key: (*) $P < 0.05$ compared with LPS + vehicle. (Right panel) Dose-related effect of rBPI$_{23}$ on serum TNF-α. Serum levels of TNF-α were compared among different rBPI$_{23}$ dose groups 90 min after initiation of LPS infusion. Key: (*) $P < 0.05$ compared with LPS + vehicle (0 mg/kg rBPI$_{23}$). All values are means ± SEM.

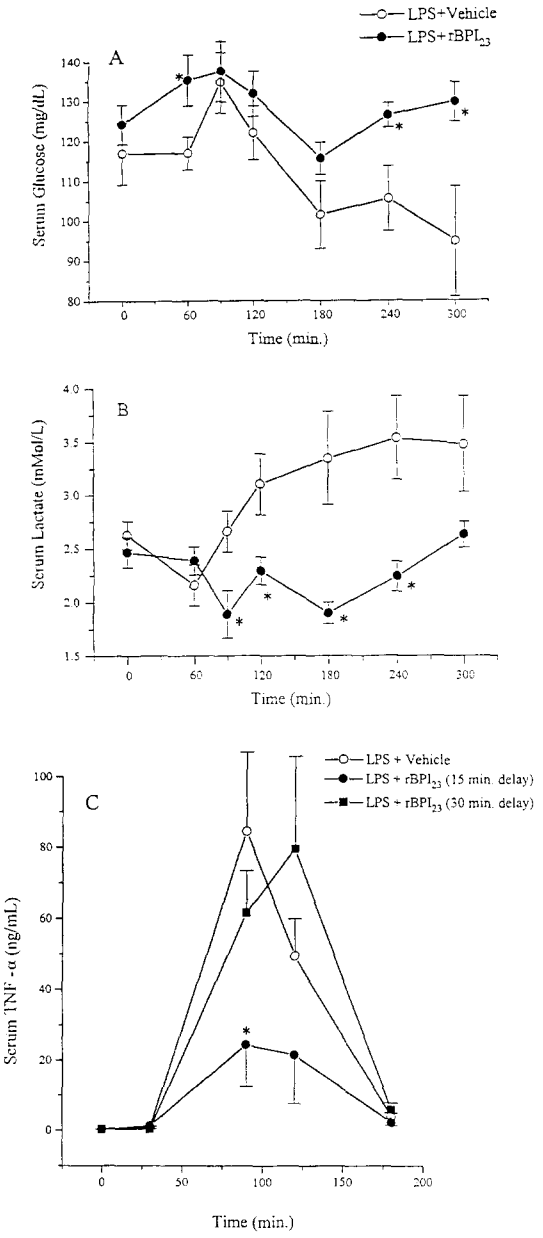

Fig. 4. (A and B) Effect of a 30-min delayed infusion of $rBPI_{23}$ on LPS-induced alterations in glucose (A) and lactate (B). Rats (8/group) were challenged with 0.25 mg/kg of LPS, and were infused with 5 mg/kg of $rBPI_{23}$ or vehicle 30 min after the initiation of LPS infusion. Key: (*) $P < 0.05$ compared with LPS + vehicle. (C) Effect of a 15- and 30-min delayed infusion of $rBPI_{23}$ on LPS-induced TNF-α elevation. LPS (0.25 mg/kg) was infused over 30 min, and $rBPI_{23}$ (3 mg/kg) was infused beginning 15 or 30 min after the initiation of LPS infusion. Key: (*) $P < 0.05$ compared with LPS + vehicle. All values are means ± SEM.

contrast, the control protein had no effect. Maximal inhibition of TNF-α elevation in serum was observed at a dose of $rBPI_{23}$ as low as 0.1 mg/kg (Fig. 3, right panel). A dose of 0.03 mg/kg had no effect.

Effect of delayed treatment with $rBPI_{23}$. When $rBPI_{23}$ infusion (5 mg/kg) was delayed for 30 min after initiation of LPS infusion, the reduction in glucose levels was significantly less severe at 240 and 300 min than in the vehicle-treated group, and there was a return to baseline levels before the end of the experiment (Fig. 4A). The elevation in lactate following LPS-infusion was inhibited significantly by the 30 min delayed infusion of $rBPI_{23}$ (Fig. 4B). In contrast, 30 min delayed infusion of $rBPI_{23}$ (3 mg/kg) did not inhibit LPS-induced TNF-α elevation (Fig. 4C). However, when $rBPI_{23}$ infusion was delayed for 15 min the TNF-α elevation was reduced significantly (Fig. 4C).

DISCUSSION

The results of the present investigation demonstrate that $rBPI_{23}$, a recombinant N-terminal fragment of BPI, can inhibit significantly alterations in serum glucose, lactate, and TNF-α elevation associated with endotoxemia. Simultaneous infusion of $rBPI_{23}$ prevented the early transient hyperglycemia and the late drop of glucose induced by LPS, whereas a control protein had no effect. Moreover, $rBPI_{23}$ inhibited LPS-induced elevations in serum lactate and TNF-α in a dose-dependent manner. These results support findings of other studies that the LPS binding and neutralizing properties of BPI reside in the N-terminal portion of the molecule [21–23, 25, 26].

Animal studies have indicated that LPS challenge results in a biphasic change in glucose. The early response is characterized by a transient and mild hyperglycemia, increased glucose turnover, gluconeogenesis, and an increase in tissue glucose oxidation [4, 16]. This hyperglycemia is generally considered to be due to large increases in blood levels of catecholamines, glucagon, and glucocorticoids [4, 16]. The late phase, on the other hand, is characterized by a profound hypoglycemia, depletion of hepatic glycogen, depression of gluconeogenesis, enhanced glucose oxidation, and probably reflects an increased insulin level as well as an enhanced insulin sensitivity [2, 28]. In animal endotoxemia models, hyperlactatemia may reflect a combination of a strongly stimulated rate of glycogen breakdown [29–31] and tissue hypoxia. Hyperglycemia has been evoked in rats with a dose of LPS as low as 0.1 mg/kg, and hyperlactatemia with a dose as low as 0.001 mg/kg [7]. In the present study, when a higher but still nonlethal dose (0.25 mg/kg) of LPS was infused over 30 min, we also observed a transient hyperglycemia followed by a decrease in glucose and a persistent increase in serum lactate. The reduction in glucose levels was less severe than previously observed in animals challenged with higher doses of LPS [4, 16]. Our results may have been influenced by the effects of anesthesia, which may have resulted in slightly elevated levels of glucose and lactate in the saline groups compared with normal conscious animals. Nevertheless, the changes in serum glucose and lactate associated with LPS challenge markedly and significantly differed from those observed in the saline control animals, and were attenuated significantly by $rBPI_{23}$ treatment.

Carbohydrate dyshomeostasis is a prominent component in endotoxin or septic shock. Elevated blood lactate concentrations have long been recognized as a consequence of tissue hypoxia [30–33]. Lactate is thought to be a major metabolic substrate in endotoxin shock [29]. The concentration of lactate determined early in shock has been shown to be a valuable aid in the assessment of the severity, prognosis and response to therapy in shock patients, a high level of lactate being associated with a high mortality rate [9–11]. Hyperglycemia is found in sepsis or early septic shock, especially in hyperdynamic septic patients [30, 31, 34–37], and hypoglycemia is often associated with severe sepsis or septic shock [8, 38–41]. To a certain extent, the pattern of glucose and lactate responses to LPS challenge observed in the present study mimics the clinical features of septicemia and septic shock. However, this animal model differs from human sepsis in that LPS is released over a much longer period of time and perhaps intermittently in human sepsis [42, 43]. Thus, although the response may be similar, the exposure to endotoxin in this experimental model is different from the clinical situation.

TNF-α, produced predominantly by activated monocytes/macrophages in response to LPS stimulation, has been implicated in the pathogenesis of the sepsis syndrome in experimental animals [12, 13, 44–46] and in humans [47–49]. TNF-α is capable of modulating glucose metabolism by itself and by interaction with other glucoregulatory mechanisms [13, 50]. In experimental animals, TNF-α administration caused lactic acidosis, transient hyperglycemia followed by hypoglycemia, hypotension and similar hormonal changes as endotoxemia [12–14]. TNF-α also influenced peripheral glucose utilization, evidenced *in vitro* by increased glucose uptake and increased lactate release [51]. However, several studies have suggested that TNF-α is not the only mediator of LPS-induced metabolic disturbances [52, 53]. Anti-TNF-α antibodies attenuated the increase in plasma lactate and glucagon levels in LPS-challenged rats but failed to prevent LPS-induced hyperglycemia [52, 53]. Other mediators such as platelet-activating factor (PAF) [15, 54] and eicosanoids [17] may also play important roles in LPS-induced carbohydrate disturbances. In the present study, $rBPI_{23}$ inhibited the LPS-induced TNF-α accumulation in the blood. These results are consistent with findings that $rBPI_{23}$ can inhibit LPS-induced production of TNF-α in whole blood [22], and in an actinomycin D-sensitized mouse model of endotoxemia [25]. We also noted that the elevation of TNF-α was maximally inhibited by $rBPI_{23}$ at a dose as low as 0.1 mg/kg, whereas the hyperglycemia and hyperlactatemia were not inhibited significantly unless a much higher dose (5 mg/kg) of $rBPI_{23}$ was used. Furthermore, when initiation of $rBPI_{23}$ infusion was delayed until immediately after the LPS infusion, there was a significant inhibition of LPS-induced alterations in lactate and glucose, but no effect on the TNF-α elevation. These data are consistent with previous findings that LPS-induced metabolic alterations in glucose and lactate are at least partially independent of TNF-α levels [53], and that additional mediators may be involved [15, 52–54].

The finding that delay of $rBPI_{23}$ treatment until after cessation of the LPS infusion still resulted in significant attenuation of the decrease in glucose and significantly reduced the elevation in lactate levels was unanticipated. Indeed, the effect on lactate levels with delayed $rBPI_{23}$ infusion was similar to that observed with simultaneous infusion. These results suggest that the glucose/lactate response to LPS required the presence of active LPS beyond the 30-min infusion. Presumably, effector cells must be exposed to LPS for longer than 30 min in order to initiate the entire response. Thus, intervention with $rBPI_{23}$, even at the end of the challenge, interrupted the ongoing cascade leading to the metabolic disturbance.

The data add to the increasing body of evidence indicating that $rBPI_{23}$ neutralizes the biological effects of LPS [20, 21] and is protective in animal models of endotoxemia [25, 26] and bacteremia [55, 56]. In the present study, $rBPI_{23}$ protected LPS-challenged rats against carbohydrate dyshomeostasis and TNF-α elevation. Delayed treatment with $rBPI_{23}$ also significantly inhibited these LPS-induced metabolic disturbances. Different dose–response profiles of $rBPI_{23}$ on LPS-induced alterations in glucose, lactate and TNF-α are consistent with previous findings that mediators other than TNF-α may be involved in LPS-induced metabolic alterations of carbohydrates.

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