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Original Research

Effect of Prophylactic Supplementation with Grape Polyphenolics on Endotoxin-Induced Serum Secretory Phospholipase A_2 Activity in Rats

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This study investigated whether dietary supplementation of polyphenolics-rich grape extract (GE) could attenuate endotoxin-induced serum secretory phospholipase A_2 ($sPLA_2$) activity, a modulator of inflammation. Male Sprague–Dawley rats were fed a control diet or the diet supplemented with polyphenolic-rich GE (100 or 300 mg/kg daily) for 3 wk prior to intraperitoneal injection of 3 or 15 mg/kg LPS. A fluorometric assay was used to measure serum $sPLA_2$ activity during a 5-d period before and after LPS injection. Body weight, hematocrit, and serum C-reactive protein level were also measured. Administration of LPS induced a rapid increase in $sPLA_2$ activity, which peaked 1 to 2 d after LPS injection and resolved to near-baseline values on days 4 to 5. Marked declines in body weight and hematocrit, increases in C-reactive protein levels, and effects on health status also occurred. GE supplementation significantly attenuated the LPS-induced increase in $sPLA_2$ activity and decline in hematocrit, but its effects on the loss of body weight and C-reactive protein levels were not significant. Among the measurements, serum $sPLA_2$ was the only marker that showed a dose-dependent response to both LPS and GE supplementation. The current findings show that oral consumption of polyphenolic-rich GE suppresses endotoxin-induced $sPLA_2$ activity.

Abbreviations: CRP, C-reactive protein; GE, grape extract; $sPLA_2$, secretory phospholipase A_2.

Dietary polyphenolics from certain fruits, vegetables, and teas have been associated with beneficial effects on health and the prevention of diseases.[28,29] Much of the research was focused on the antioxidant properties of these compounds,[38] but it has become increasingly clear that polyphenolics can affect physiologic and pathologic processes independently from antioxidant mechanisms. More recently, particular attention has been given to the ability of polyphenolics to modulate immune processes.[4,36]

Despite ample evidence showing the overall health benefits of polyphenols, clinical studies examining the antiinflammatory effects of polyphenolics are scarce, in part because of the lack of reliable biomarkers for accessing inflammation in this context. Most in vivo or ex vivo studies of the effects of nutritional factors in the context of infections rely on the determination of cytokine levels as indicators of ongoing inflammation. Cytokines primarily are involved in the initial phase of inflammation, and most of them spike within the first few hours after the induction of inflammation and rapidly return to baseline levels thereafter.[5,10,11,20,39] Therefore, determination of cytokine levels alone may not quantitatively reflect the extent of inflammatory response, which often lasts from days to weeks or even months, in association with significant pathologic changes that occur over an extended period of time. This pattern suggests that cytokine levels may not provide an accurate and consistent reflection of the effects of nutritional factors on inflammation. However, cytokines further activate various target cells and trigger the synthesis and release of a number of acute-phase response proteins including the C-reactive protein (CRP) produced by the liver[3,31] and secretory phospholipase A_2 ($sPLA_2$), which is expressed by several types of immune cells.[44,46] The levels of these proteins increase markedly in the circulating blood under conditions of acute inflammation, such as those in patients with bacterial infections and trauma.[25,33] Therefore, high serum levels of CRP and $sPLA_2$ have been recognized as important risk markers in infection and cardiovascular disease.[8,33,48] The purpose of the current study was to investigate whether supplementation of polyphenolics-rich grape extract (GE) suppressed the endotoxin-induced inflammatory response, as determined by measuring $sPLA_2$ activity in serum.

Materials and Methods

Materials. L-α-phosphatidylglycerol and LPS (derived from *E. coli* 055:B5) phenol extract were purchased from Sigma-Aldrich Chemical (St Louis, MO). 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-undecanoyl)-sn-glycero-3-phosphocholine was obtained from Molecular Probes (Invitrogen, Carlsbad, CA). Powdered grape seed and skin extract (GE; Provex CV) was obtained from Melaleuca (Idaho Falls, ID).

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Experimental design. The use of rats for this investigation conformed to guidelines for the care and use of laboratory animals set by the University of Wisconsin Research Animal Resource Center and the NIH. The studies were performed by using 2-mo-old, male, Hsd:Sprague–Dawley outbred rats (Harlan, Madison, WI) weighing approximately 250 g. The animals were free of rat coronavirus and internal and external parasites at the time of study. Rat parvoviruses, rat theiloviruses, *Pneumocystis* spp., and common bacteria were not excluded from the rats in this facility. The rats were housed in solid-bottom cages containing corncob contact bedding and had ad libitum access to hyperchlorinated water (3.0 to 7.0 ppm) supplied via automatic watering manifold to each individual cage. The rooms were maintained at 64 to 79 °F (20.6 to 23.3 °C), at 30% to 70% humidity, and under 12:12-h light:dark cycle.

A total of 36 rats were fed a powdered control diet (65 g/d; 8728C Teklad Certified Rodent Diet, Harlan) for a week prior to their use in experiments. After this 1-wk acclimation period, the rats were randomly assigned to 3 groups and fed a control diet supplemented with 0, 100, or 300 mg GE/kg daily for 3 wk. At the end of this period (denoted as day 0), the rats in each group were weighed, divided randomly into 2 groups, and injected intraperitoneally with either 3 or 15 mg/kg LPS (diluted in 1 mL sterile water). Blood samples were collected from the rats on day 0 (preLPS baseline) and then on days 1 through 5 after LPS injection; body weights were recorded prior to each blood collection. The rats were maintained on their appropriate diets during the 5 d after LPS injection. The blood samples (0.1 mL) were obtained via the saphenous vein from anesthetized (5% isoflurane for induction, 3% isoflurane for maintenance) rats and collected into BD Micro-Fine Tubes with clot activator (Fisher Scientific, Pittsburgh, PA). The blood was centrifuged at $2000 \times g$ for 10 min to obtain serum, which was stored at −70 °C for subsequent analysis. Additional blood was collected in heparinized microhematocrit tubes (Fisher Scientific) for hematocrit analysis.

Determination of serum $sPLA_2$ activity and CRP concentration. Serum $sPLA_2$ activity was determined by using a slightly modified version of a high-throughput microplate fluorescent assay that we recently developed.[42] Briefly, 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-undecanoyl)-sn-glycero-3-phosphocholine (0.014 mg) and L-α-phosphatidylglycerol (2 mg) were mixed together in chloroform. The chloroform was evaporated off, and the free phospholipids were dissolved in 1 mL 100% ethanol to form the stock substrate solution. The stock solution was stored at −20 °C, and was found to be stable for at least 1 mo. To determine $sPLA_2$ activity in a serum sample, an assay reaction mixture was freshly prepared in a glass tube kept on ice. The reaction mixture contained 10 µL stock substrate—ethanol solution (20 µg phospholipids) and 3.3 µL serum in a final volume of 1 mL assay buffer (0.01 M Tris-HCl [pH 7.4] and 10 mM Ca^{2+}). The mixture was vortexed, and aliquots (300 µL) were transferred promptly in triplicate to a white polystyrene 96-well microplate (Porvair PS White, PerkinElmer, Waltham, MA). The microplate was immediately placed in a temperature-controlled (30 °C) spectrometer (LS50B Luminescence Spectrometer, PerkinElmer) with a microplate reader attachment. The fluorescence intensity in each well was recorded every 10 s for 60 cycles at 488 nm excitation (excitation slit, 2.5 nm) and 530 nm emission (emission slit, 5.0 nm). Ca^{2+}-independent hydrolysis of substrate was determined by performing the assay in the presence of 20 mM EGTA in the assay buffer. The $sPLA_2$ activity (fluorescence intensity /min/µL serum) was determined after Ca^{2+}-independent background was subtracted, as previously described.[42] Serum CRP levels were determined by using a rat CRP ELISA kit (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's protocol.

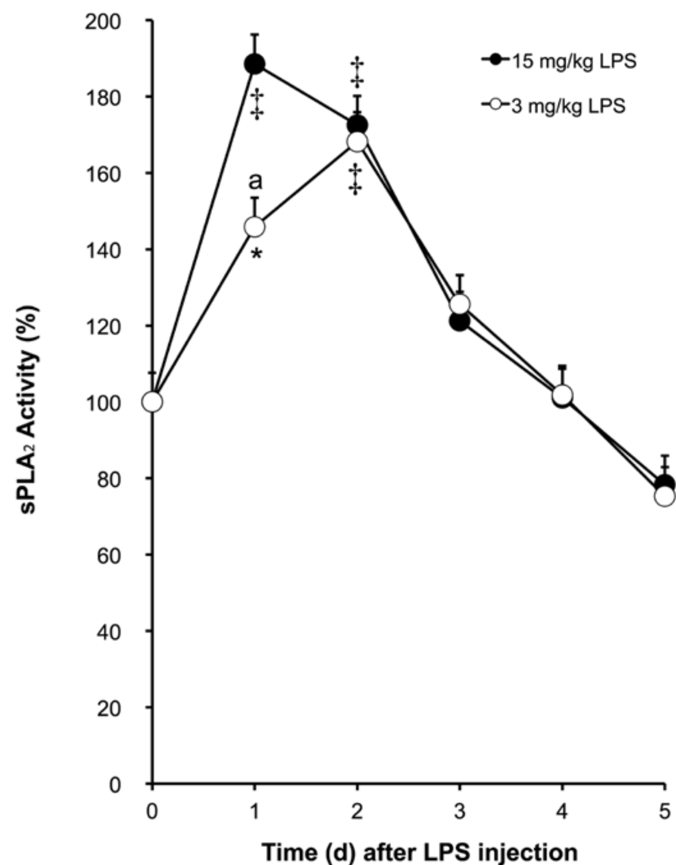

Figure 1. Changes in serum $sPLA_2$ activity (expressed as a percentage of the day 0 activity) after intraperitoneal administration of 3 or 15 mg/kg LPS to rats ($n = 6$ per group) fed a control diet. Significant (*, $P \leq 0.05$; ‡, $P \leq 0.001$) difference when compared with value for day 0 within the same group; significant (a, $P \leq 0.05$) difference between groups at a particular time point.

Assessment of health status. The health status of the rats after the LPS injection were assessed by a veterinarian or veterinary staff who were blinded to the diet and LPS dose that the animals received. In addition to a commonly used body condition scoring system,[43] the rats were scored on predefined characteristics that included the assessment of grooming behavior, porphyrin staining around eyes, opening of eyes, posture, respiration, fecal output, perianal area, level of activity, and hydration status.

Statistical analysis. A repeated-measures ANOVA model, which included factors of time, LPS dose, and diet group, was fit for each measured parameter by using the Proc Mixed function of the statistical software (SAS Software v8, SAS Institute, Cary, NC). All appropriate second- and third-order interactions between these factors also were included in the model. The correlations between observations taken on the same rat over time were modeled by using a compound symmetry correlation structure. For each measured marker, the model was fit by using the untransformed data, and the residuals were evaluated to ensure that standard ANOVA

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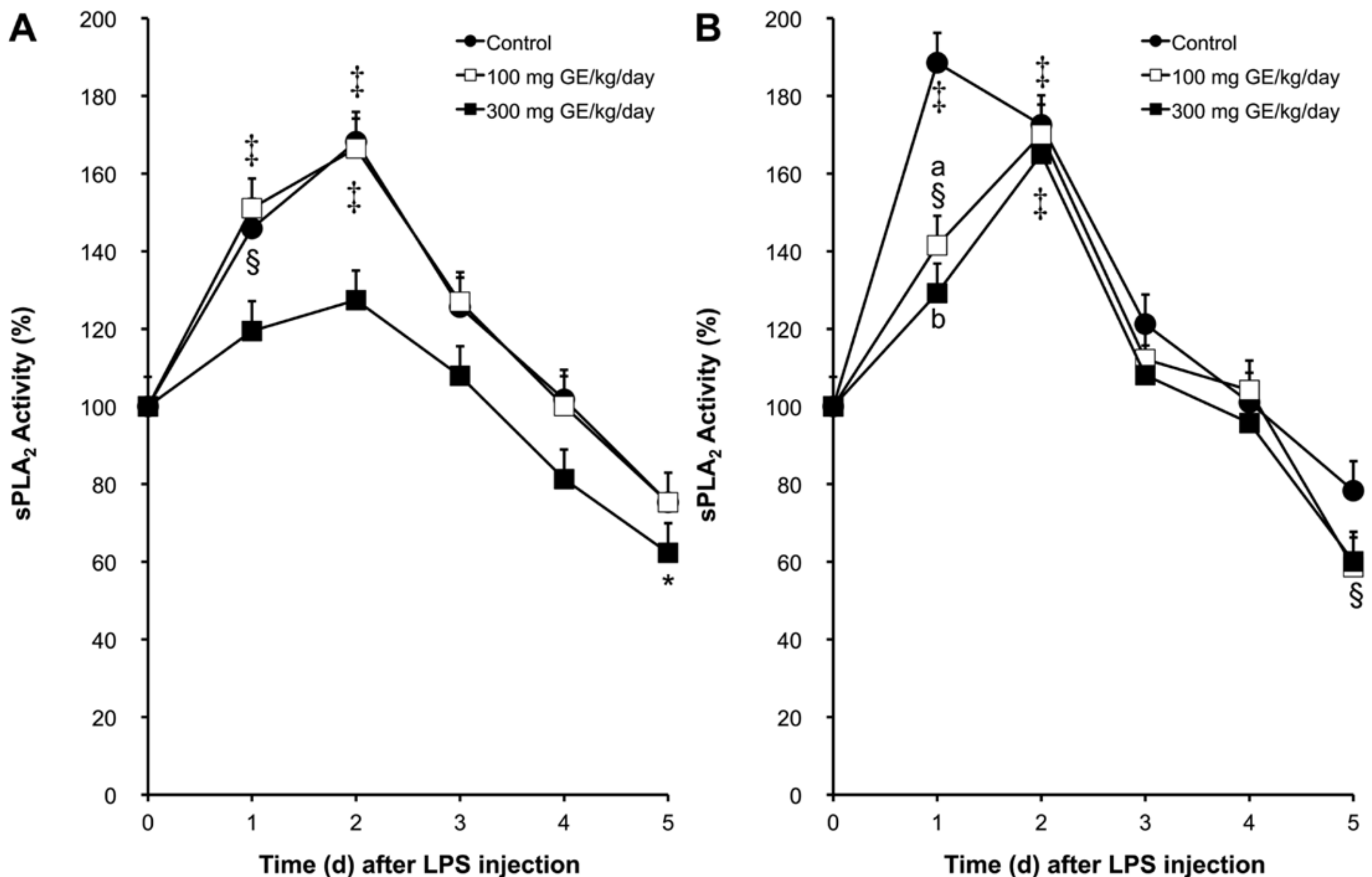

Figure 2. Effect of LPS administration and GE supplementation on serum $sPLA_2$ activity. The figure shows the changes in serum $sPLA_2$ activity following intraperitoneal administration of (A) 3 mg/kg LPS and (B) 15 mg/kg LPS to rats ($n = 6$ per diet) fed a control diet or the control diet supplemented with 100 or 300 mg/kg GE daily. Significant (*, $P \leq 0.05$; §, $P \leq 0.005$; and ‡, $P \leq 0.001$) difference when compared with the value for day 0 within the same group; significant (a, $P \leq 0.05$; b, $P \leq 0.001$) difference between values for control group and each GE-supplemented group at a particular time point.

assumptions of constant variance and normality were reasonably met. Transformations of the data were performed when required to improve adherence to these assumptions. Once a good fit was achieved, type III tests were performed to evaluate the significance of the effects of interest for each measured marker, and least-square means were calculated for each factor combination. Any least-square means comparisons made subsequent to the type III tests were adjusted by using the Tukey–Kramer P-value adjustment. The data are reported as least-square mean ± SEM. Statistical significance was defined as a P value of less than 0.05.

Results

Serum $sPLA_2$ activity. Administration of LPS induced a marked increase (70% to 90% above baseline) in serum $sPLA_2$ activity in the control diet-fed rats (Figure 1) on days 1 and 2 after LPS injection. The effects of the 2 doses of LPS were different on day 1 but were nearly identical on days 2 through 5 after LPS injection. On day 1, serum $sPLA_2$ activity was approximately 30% greater ($P <$ 0.05) in rats that received the higher dose.

Supplementation of the diet with 300 mg/kg GE daily inhibited ($P < 0.05$) the increases in $sPLA_2$ activity induced by 3 mg/kg LPS (Figure 2 A). In contrast, 100 mg/kg GE daily had no effect. In the rats given 15 mg/kg LPS, both 100 ($P < 0.05$) and 300 ($P <$ 0.001) mg/kg GE inhibited the LPS-induced increase in $sPLA_2$ activity on day 1 (Figure 2 B).

Body weight. The administration of LPS to the control-fed rats resulted in loss of body weight; rats weighed least on day 2 and then their weights stabilized between days 3 through 5 (Figure 3). The magnitude and rate of decline in body weight was LPS dose-dependent. The higher dose of LPS produced 50% greater ($P < 0.001$) loss in body weight than did the lower dose. Supplementation of the control diet with GE did not significantly affect the observed changes in body weight induced by LPS (Figure 4 A and B).

Hematocrit. Hematocrit declined after the administration of LPS to the control-fed rats, with the change (approximately 20% decline by day 5) becoming statistically significant ($P < 0.001$) on days 3 and 4 (Figures 5 A and B). There was no discernible difference in the effects produced by the 2 doses of LPS. The supplementation of the control diet with 100 or 300 mg/kg GE daily significantly ($P < 0.05$) attenuated the effect of 3 mg/kg LPS on hematocrit (Figure 5 A). However, neither of the supplemented diets had any effect on the changes induced by the higher dose of LPS (Figure 5 B).

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Serum CRP level. Administration of LPS produced significantly ($P < 0.001$) increased serum CRP levels in the control diet-fed rats on day 2 (Figure 6 A and B). There was no significant difference between the effects of the 2 LPS doses on CRP concentrations in serum. Changes in CRP levels were more variable than were changes in $sPLA_2$ activity, thus reducing the statistical power to detect an effect of GE supplementation.

Assessment of health status. The injection of LPS caused deterioration in the health status of the rats on either the control or GE-supplemented diet. These effects of LPS were noticeable within a few hours after administration, and the greatest deterioration was present at 24 to 48 h after injection. The magnitude of effect was greater in rats that received the higher dose of LPS. In general, these rats showed reduced grooming behavior, partial squinting of eyes, slightly hunched posture, fecal staining or soft feces around the perianal area, and reduced activity (that is, rats moved only when prompted). Rats on the GE-supplemented diets appeared to be less affected, but this characteristic could not be quantitated sufficiently. Regardless of diet, all rats returned to normal body movement by 3 to 4 d after LPS injection.

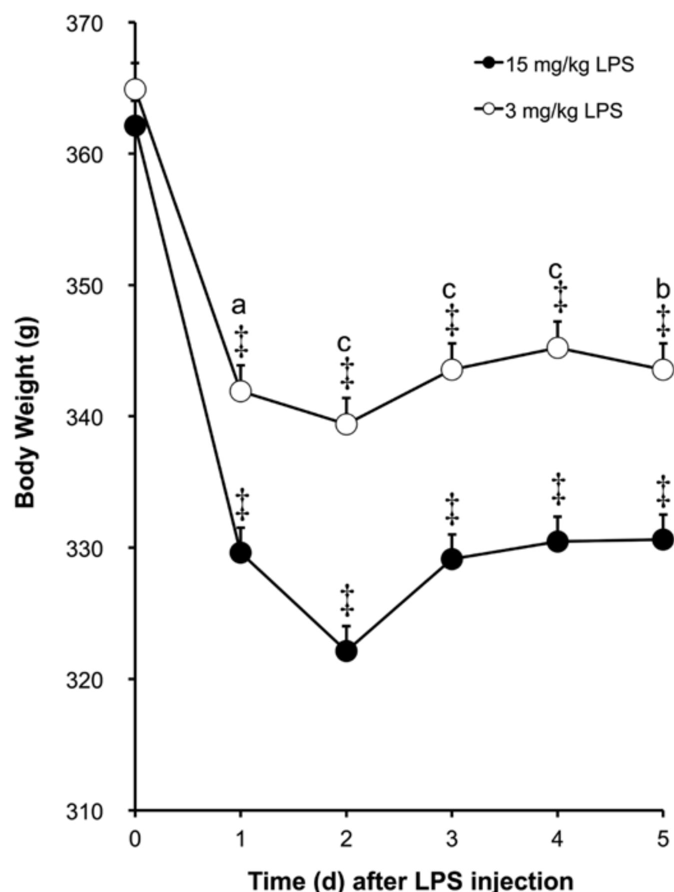

Figure 3. Effect of LPS administration on body weight. The figure shows the changes in body weight after intraperitoneal administration of 3 or 15 mg/kg LPS to rats ($n = 6$ per dose) fed a control diet. Significant (‡, $P \leq 0.001$) difference when compared with the value for day 0 within the same group; significant (a, $P \leq 0.05$; b, $P \leq 0.005$; c, $P \leq 0.001$) difference between groups at a particular time point.

Discussion

The administration of LPS, an endotoxin derived from bacterial membranes, to animals is widely used to study the pathophysiologic consequences of endotoxemia and sepsis. Although $sPLA_2$ plays a central role in the systemic immune response[45,46] and although its level increases in sera of volunteer subjects after receiving an intravenous infusion of LPS[34] and in rabbits after LPS injection,[44] the time-course of changes in serum $sPLA_2$ activity after LPS administration has not been studied thoroughly. The highly sensitive high-throughput fluorometric assay that we previously developed[42] allows us to efficiently study the time-course of $sPLA_2$ activity changes and the effects of therapeutic strategies. Furthermore, in the current study, modifications to the previously described assay substrate preparation[42] have resulted in a 50% increase in the sensitivity of the assay (data not shown). In the current study, intraperitoneal injection of LPS induced a highly reproducible profile of serum $sPLA_2$ activity, characterized by a rapid increase with an LPS dose-dependent peak 1 to 2 d after injection, followed by a resolution to near-baseline values over a 5-d period (Figure 1). The $sPLA_2$ assay showed clear differences in serum $sPLA_2$ activities of the rats injected with 3 compared with 15 mg/kg LPS on day 1, the day on which the rats showed the greatest deterioration. The $sPLA_2$ assay also clearly demonstrated the rise and fall of serum $sPLA_2$ activity in accordance with the onset and recovery of LPS-induced illness during the 5-d period. These observations are consistent with a study of intravenous infusion of endotoxin in volunteer subjects that reported a maximal increase in $sPLA_2$ activity occurred 24 h after infusion[34] and are similar to the pattern of serum $sPLA_2$ levels in a patient during treatment for sepsis.[19]

Production of $sPLA_2$ can be stimulated by TNF alone or in concert with other cytokines such as interleukin-1.[45,46] TNF levels decline within 2 to 4 h after LPS administration.[10,20,34] However, $sPLA_2$ and the pathways that $sPLA_2$ triggers drive the pathophysiologic process forward. Of the many isoforms of PLA_2s,[7] only a few are found circulating in serum. Of these, $sPLA_2$-IIA (14 kDa) is the predominant form involved in inflammatory immune responses;[13,22,45] this isoform also may play a key role in antimicrobial defense[23] and regulate the synthesis of bioactive lipid mediators that have a broad range of physiologic and pathologic effects.[6] The persistent presence of $sPLA_2$-IIA like that in the sera of patients with systemic inflammation[1] and bacteremia[37] may exacerbate and intensify inflammation.

The source of $sPLA_2$-IIA in serum is still unknown. Although cells with secretory granules, such as macrophages, platelets, neutrophils, and mast cells, have traditionally been implicated as contributors,[16,17,21,26,27] Paneth cells of the gastrointestinal tract produce the greatest amounts of $sPLA_2$-IIA in humans.[24] Mouse small intestine luminal fluid also contains high quantities of $sPLA_2$-IIA.[32] Similarly in rats, $sPLA_2$-IIA is synthesized primarily by the Paneth cells of the small intestine and the cecum.[27] A recent study illustrated that intraperitoneal injection of LPS induces a flux of $sPLA_2$ into the gastrointestinal lumen within 5 h, resulting in the degradation of the phosphatidylcholine-rich protective hydrophobic gastrointestinal barrier.[50] The degradation of the mucosal barrier correlated with an increase in lysophosphatidylcholine, an injurious byproduct of phosphatidylcholine hydrolysis. Consequently, an increase in gastrointestinal permeability was observed[50] and well characterized.[9] Oral delivery of a $sPLA_2$-IIA inhibitor attenuated the luminal $sPLA_2$ activity and prevented changes in GI permeability.[50] Previously, lysophosphatidylcholine

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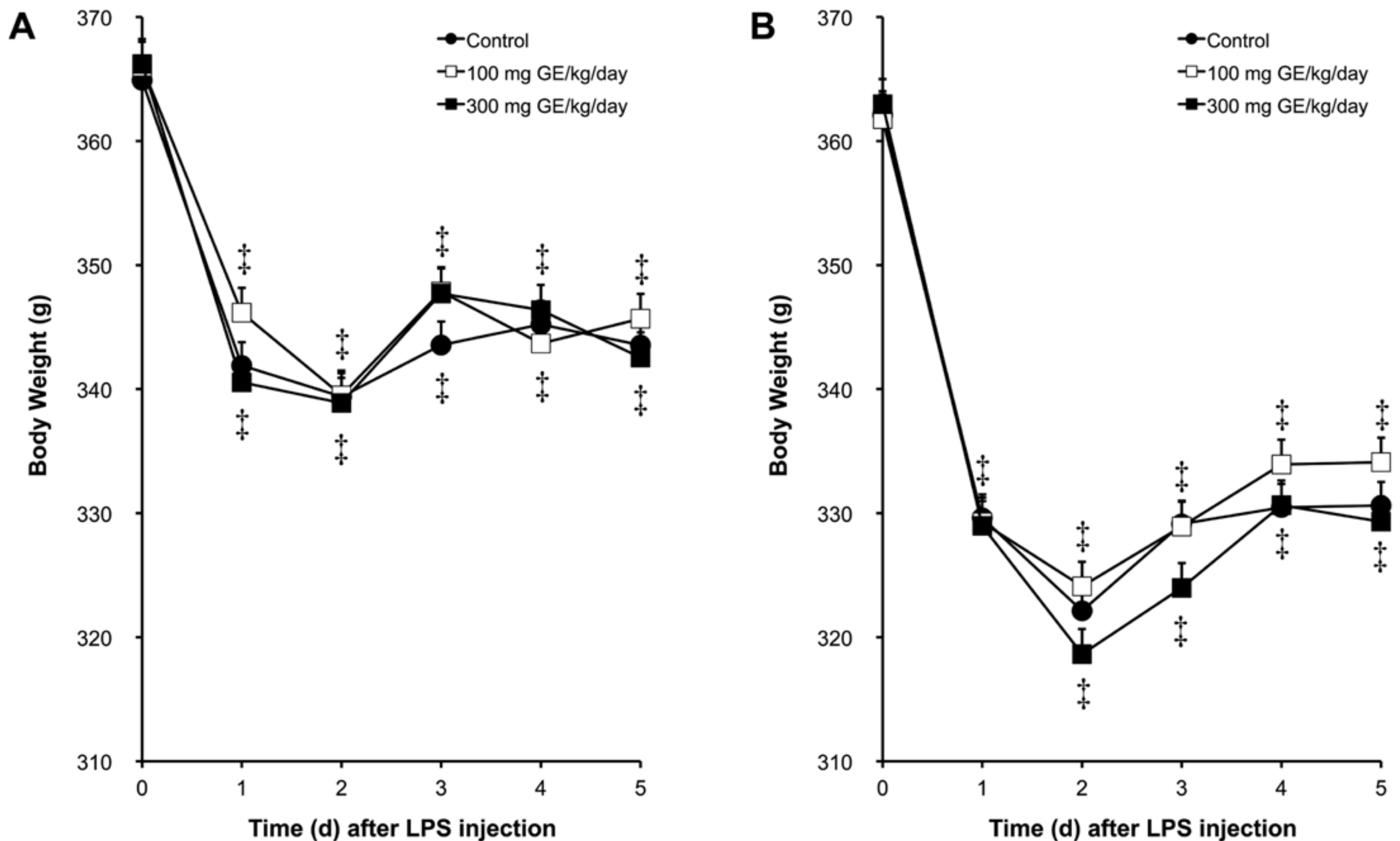

Figure 4. Effect of LPS administration and GE supplementation on body weight. The figure shows the changes in body weight after intraperitoneal administration of (A) 3 mg/kg LPS and (B) 15 mg/kg LPS to rats (n = 6 per diet) fed a control diet or the control diet supplemented with 100 or 300 mg/kg GE daily. Significant (‡, $P \leq 0.001$) difference when compared with the value for day 0 within the same group.

was shown to increase the translocation of luminal bacteria,[40] suggesting that a spike in lysophosphatidylcholine during sepsis may drive an influx of immunogenic material into the body. In addition, the increased permeability may allow for the entry of luminal $sPLA_2$-IIA into circulation, accounting for the delayed spike in serum $sPLA_2$-IIA that occurs 24 h after LPS injection. Furthermore, in a recent study, we presented evidence that the gastrointestinal tissue or vasculature may be an important source of serum $sPLA_2$ and that this source may be regulated independently from luminal sources of $sPLA_2$, such as Paneth cells.[32] It is unknown whether dose and time after induction influence the contributions of various sources of $sPLA_2$ to its systemic activity during LPS challenge. This lack of knowledge presents a challenge in interpreting various aspects of the effects of GE on serum $sPLA_2$ activity.

In the current study, the higher dose of GE supplementation significantly inhibited the changes in serum $sPLA_2$ activity induced by the lower dose of LPS. However, under the higher dose of LPS, the higher dose of GE inhibited serum $sPLA_2$ activity only on day 1 after induction. Surprisingly, the lower dose of GE supplementation also inhibited serum $sPLA_2$ activity at the same time point, whereas it had no effect on $sPLA_2$ activity changes induced by the lower dose of LPS at any time point. When we compare the serum $sPLA_2$ activity profile under the 2 doses of LPS, the day 1 time point was the only point at which the profile differed and was proportionate to the LPS dose. We speculate that the difference in $sPLA_2$ at this time point might be due to contributions from additional sources of $sPLA_2$ or to a secondary mechanism of induction, either of which may have been susceptible to inhibition by a lower dose of GE.

The polyphenolics in GE are largely composed of polymeric polygalloyl polyflavan-3-ols[41] that are poorly bioavailable when consumed orally.[18,35,49] Regardless, the current and other[14,15] studies have shown an influence of GE polyphenolics on modulators of inflammation. This finding raises the question whether the observed effects of GE were at least in part due to the indirect modulation of serum $sPLA_2$ activity at the gastrointestinal level. Perhaps this mechanism also explains why we did not see an effect of GE supplementation on the LPS-induced changes in CRP, which were similar[30] to those reported by others. Further studies are required to explore the source and mechanism of induction of $sPLA_2$ after LPS challenge and how these factors may be influenced by polyphenolics that are largely unabsorbed.

In addition to the health status changes, the changes in serum $sPLA_2$ activity and the increased serum CRP levels, LPS administration also resulted in the loss of body weight and decreased hematocrit. The rats did not fully recover their body weight even 5 d later, although their mobility and food intake appeared normal on day 5. The lack of recovery of body weight and packed blood cell volume changes indicate that a longer time period is needed for full recovery.

The current study has several limitations. First, we did not measure additional markers of pathophysiologic status or perform

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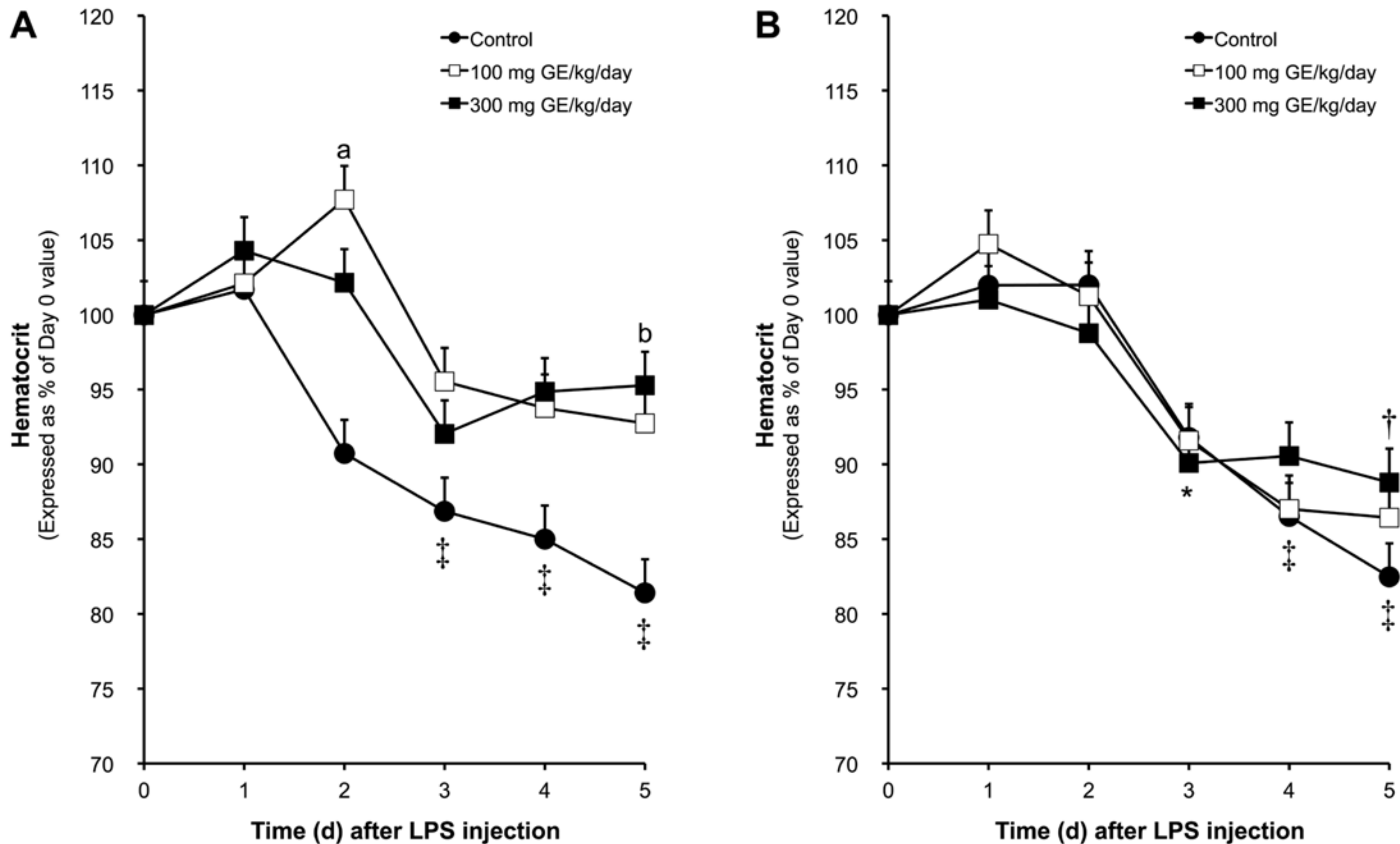

Figure 5. Effect of LPS administration and GE supplementation on hematocrit. The figure shows the changes in hematocrit after intraperitoneal administration of (A) 3 mg/kg LPS and (B) 15 mg/kg LPS to rats ($n = 6$ per diet) fed a control diet and or the control diet supplemented with 100 or 300 mg/kg GE daily. Significant (*, $P \leq 0.05$; †, $P \leq 0.01$; and ‡, $P \leq 0.001$) difference when compared with value for day 0 within the same group; significant (a, $P \leq 0.05$; b, $P \leq 0.001$) difference between values for control group and each GE-supplemented group at a particular time point.

organ histopathology, especially of the liver and kidney, that may have provided explanations for the body weight and hematocrit changes that we noted. For example, the continuous reduction of blood cell volume suggests that rats may have been overhydrated, presumably due to LPS-induced renal inflammation.[2] However, given the experimental design, the reduction in hematocrit may have resulted from the cumulative effects of the blood samplings from the animals. Additional clinical chemistry, hematology, and histopathology may have provided more conclusive statements regarding these observations. Second, we did not closely monitor changes in appetite and food intake throughout the course of the 5-d infection period that may have accounted for changes in body weight. Third, we question the efficacy of CRP as a sensitive marker of the acute-phase response in rats. CRP has been noted to be a poorly sensitive marker for the acute-phase response in rats, with variable serum concentrations.[47] In addition, healthy rodents have a higher baseline level of CRP compared with nonrodent species and humans.[12]

In summary, LPS administration generated noticeable deterioration in the health status, caused declines in body weight and hematocrit, and increased serum $sPLA_2$ activity and CRP level in our rats. Dietary supplementation with GE attenuated the LPS-induced increase in $sPLA_2$ activity and decrease in hematocrit but had little effect on the loss of body weight and serum CRP level. This study provides evidence that the oral consumption of polyphenolics-rich GE may suppress endotoxin-induced $sPLA_2$ activity and that such an effect can be determined by using our serum $sPLA_2$ activity assay. The determination of $sPLA_2$ activity may be a useful tool for examining the effects of polyphenolic-rich supplements in clinical studies.

Acknowledgments

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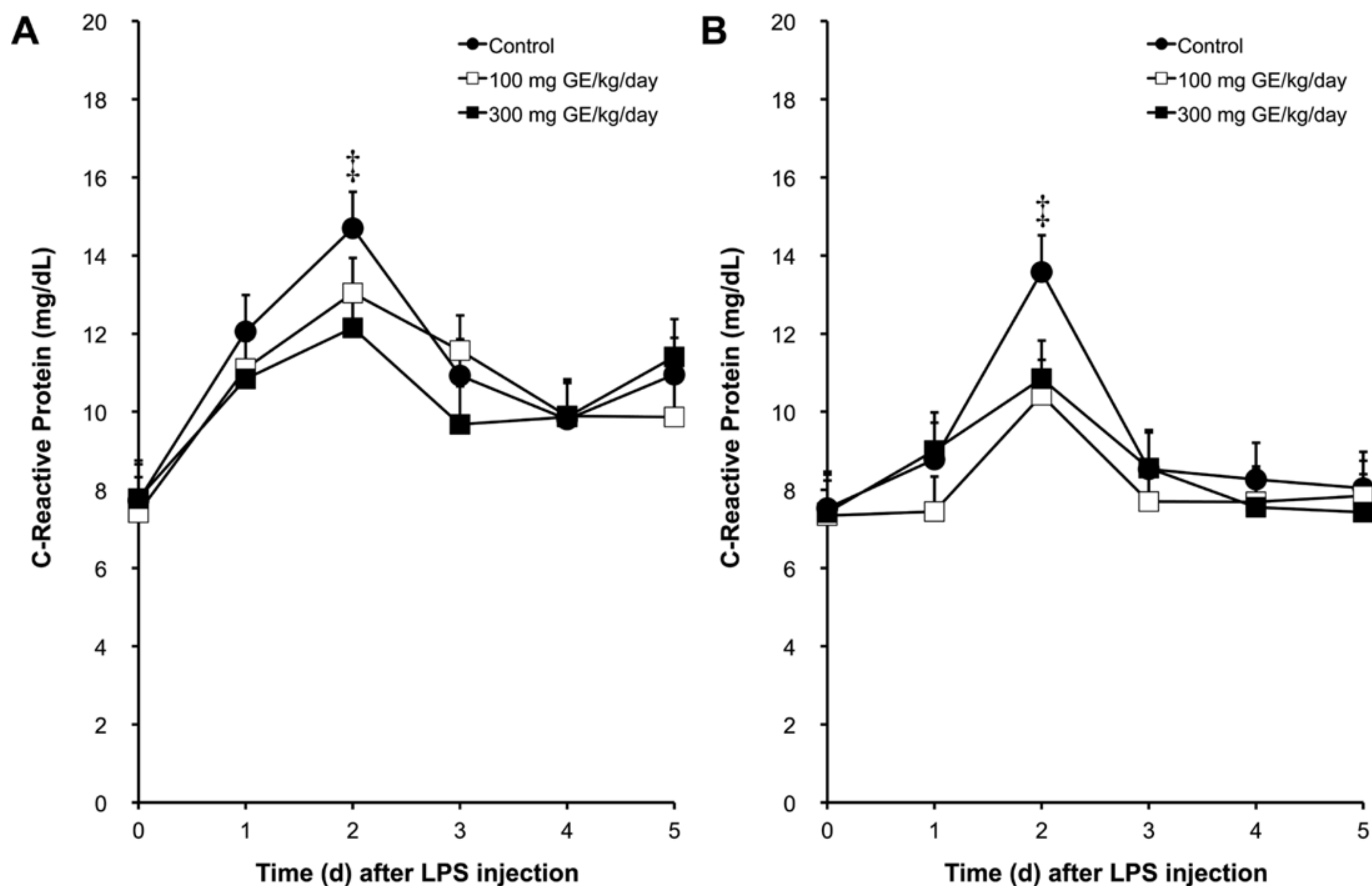

Figure 6. Effect of LPS administration and GE supplementation on serum CRP levels. The figure shows the changes in serum CRP levels after intraperitoneal administration of (A) 3 mg/kg LPS and (B) 15 mg/kg LPS to rats (n = 6 per diet) fed a control diet or the control diet supplemented with 100 or 300 mg/kg GE daily. Significant (‡, $P \le 0.001$) difference compared with value for day 0 within the same group.

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