Ethyl gallate, a scavenger of hydrogen peroxide that inhibits lysozyme-induced hydrogen peroxide signaling in vitro, reverses hypotension in canine septic shock

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Submitted 15 April 2010; accepted in final form 8 November 2010

Mink SN, Jacobs H, Gotes J, Kustin K, Cheng ZQ. Ethyl gallate, a scavenger of hydrogen peroxide that inhibits lysozyme-induced hydrogen peroxide signaling in vitro, reverses hypotension in canine septic shock. J Appl Physiol 110: 359–374, 2011. First published November 11, 2010; doi:10.1152/japplphysiol.00411.2010.—Although hydrogen peroxide (H_{2}O_{2}) is a well-described reactive oxygen species that is known for its cytotoxic effects and associated tissue injury, H_{2}O_{2} has recently been established as an important signaling molecule. We previously demonstrated that lysozyme (Lzm-S), a mediator of sepsis that is released from leukocytes, could produce vasodilation in a phenylephrine-constricted carotid artery preparation by H_{2}O_{2} signaling. We found that Lzm-S could intrinsically generate H_{2}O_{2} and that this generation activated H_{2}O_{2}-dependent pathways. In the present study, we used this carotid artery preparation as a bioassay to define those antioxidants that could inhibit Lzm-S's vasodilatory effect. We then determined whether this antioxidant could reverse the hypotension that developed in an Escherichia coli bacteremic model. Of the many antioxidants tested, we found that ethyl gallate (EG), a nonflavonoid phenolic compound, was favorable in inhibiting Lzm-S-induced vasodilation. In our E. coli model, we found that EG reversed the hypotension that developed in this model and attenuated end-organ dysfunction. By fluorometric H_{2}O_{2} assay and electrochemical probe techniques, we showed that EG could scavenge H_{2}O_{2} and that it could reduce H_{2}O_{2} production in model systems. These results show that EG, an antioxidant that was found to scavenge H_{2}O_{2} in vitro, was able to attenuate cardiovascular dysfunction in a canine in vivo preparation. Antioxidants such as EG may be useful in the treatment of hemodynamic deterioration in septic shock.

IN SEPTIC SHOCK, hydrogen peroxide (H_{2}O_{2}) is a well-described reactive oxygen species that is known for its cytotoxic effects and associated tissue injury (6). In recent years, however, H_{2}O_{2} has also been established to be an important regulator of eukaryotic signal transduction (2, 6, 41). In multicellular organisms, H_{2}O_{2} has been shown to activate signaling pathways to stimulate cell proliferation, differentiation, migration, apoptosis, and vascular function. H_{2}O_{2} is generated in response to various stimuli, including cytokines and growth factors, from superoxide anion, which is produced by the partial reduction of oxygen by cytochrome-c oxidase in mitochondria, and from membrane-associated NADPH oxidases among other mechanisms (6). In a newly described process, Wentworth and colleagues (35, 43–46) showed that antibodies, regardless of source or antigenic specificity, can intrinsically generate H_{2}O_{2} from singlet oxygen (1O_{2}*-). They further demonstrated that this process is catalytic and that antibodies use H_{2}O as an electron source in reactions that eventually lead to the formation of H_{2}O_{2}. Wentworth and colleagues also showed that other proteins, such as chick egg ovalbumin and β-lactoglobulin, were capable of intrinsically producing H_{2}O_{2} (43).

Of the many nonimmunoglobulin proteins that are released in septic shock and that could possibly generate H_{2}O_{2}, we previously found that lysozyme (Lzm-S) could also intrinsically generate H_{2}O_{2} (24). Lzm-S is released from leukocytes of the spleen and other organs in sepsis (29) and has been shown to produce vasodilation and myocardial depression in canine models of septic shock (19, 24–30). To produce myocardial depression, Lzm-S binds to the endocardial endothelium (EE). This binding results in the endocardial release of nitric oxide (NO) and the subsequent activation of the myocardial guanylyl cyclase pathway. We further demonstrated that this binding by Lzm-S to the EE can be inhibited in both in vitro and in vivo preparations by compounds known to inhibit Lzm-S's glycoaldehyde site (19, 30), such as N,N'-N'-triacycliclitohitroisde and N,N'-diacycliclitohitribose (39). On the other hand, Lzm-S-induced vasodilation is endothelium- and NO independent (27). Although Lzm-S still activates smooth muscle guanylyl cyclase to cause vasodilation, this effect cannot be prevented by the above competitive inhibitors of Lzm-S's active site. The reason for the different binding properties of Lzm-S in the two conditions is not yet clear.

Since systemic vasodilation is a significant factor leading to the hypotension that occurs in septic shock (1), it would be important to identify those compounds that could also block Lzm-S's vasodilatory activity in addition to inhibiting Lzm-S's myocardial depressant effect. In the present study, we tried to identify inhibitors of Lzm-S's vasodilatory activity in our phenylephrine-constricted carotid artery preparation. Specifically, we focused on those compounds that would interfere with the ability of Lzm-S to generate H_{2}O_{2}. We then determined whether this substance could reverse the systemic vasodilation that occurs in our canine Escherichia coli model of septic shock.

METHODS

The canine experiments were approved by the University Animal Care Committee and conform with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (33).

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In initial experiments, a carotid artery ring preparation was used as a bioassay to determine those compounds that could inhibit Lzm-S-induced vasodilation (27). Internal carotid artery segments (4-cm length) were removed from mongrel dogs (15–25 kg) that were previously anesthetized with pentobarbital (45 mg/kg). The arteries were dissected free from the surrounding tissues and cut to obtain rings ~4 mm in length and 4 mm in outer diameter. The rings were stretched to optimal length (~4 g) and placed into a 10-ml organ bath filled with HEPES-buffered physiological solution (in mmol/l: 118 NaCl, 2.5 CaCl2, 1.2 MgCl2, 1.4 KH2PO4, 4.9 KCl, 25 HEPES, and 11 glucose). The bath was bubbled with 100% O2 and set at 37°C with a pH of 7.35. In this protocol, the carotid rings were initially preconstricted with phenylephrine (10−5 mol/l) (27), after which measurements were determined at ~20 min after phenylephrine instillation when a stable plateau had been reached. In this study, measurements were obtained at 5 min, 15 min, and 30 min after Lzm-S instillation (see below).

Lzm-S Preparation

The human Lzm-S that was used in this study was obtained from EMD Biosciences (San Diego, CA). This preparation has only NaCl and sodium acetate buffer added to the lyophilized preparation. In previous experiments, there was no evidence to suggest that substances other than Lzm-S were present in our human and canine Lzm-S preparations to cause vasodilation in the carotid artery ring preparation (24, 27). The plasma concentration of Lzm-S measured in our in vivo sepsis model is ~10−6 mol/l, and this approximate amount of human Lzm-S (1.2 × 10−6 mol/l) was used in this organ bath preparation. Since it was previously found that Lzm-S could vasodilate very small constricted mesenteric arteries (~1-mm diameter) as well, the findings in the carotid artery preparation would be representative of what is found in the smaller arteries.

Preliminary Screened Compounds to Inhibit Lzm-S-Induced Vasodilation

The various antioxidant compounds that were initially found to be without effect included limonene, quercetin dihydrate, guaiacol, squalene, curcumin, ellagic acid, epicatechin gallate from green tea, catechin gallate, epigallocatechin gallate from green tea, polyphenol 60 from green tea, crocin, luteolin, sodium ascorbate, N-acetylcysteine (NAC), reduced glutathione, gallic acid, propyl gallate, sodium benzoate, and vitamin E. In contrast, the nonflavonoid phenolic and related compounds that included ethyl gallate (EG), methyl gallate, and ethyl 3,4-dihydroxybenzoate all blocked the effect of Lzm-S on producing vasodilation in this carotid artery preparation (all from Sigma, Oakville, ON, Canada). In this case, a dose-response relationship was determined for these latter compounds in the carotid organ bath preparation. Because EG (see Fig. 9 in discussion) seemed to have the best profile in terms of preventing Lzm-S-induced effects, this compound was used in our right ventricular trabecular (RVT) in vitro study and in our in vivo study.

To Determine Whether Ethyl Gallate Can Also Inhibit Myocardial Depression in RVT Preparation

This RVT organ bath preparation has been described previously (14, 24). Briefly, hearts were removed from mongrel dogs (15–25 kg) previously anesthetized with pentobarbital. Three or four thin right ventricular trabeculae (~1-mm diameter; 3.5-mm length) were placed into respective 5-ml organ baths. The baths were maintained at 37°C, were bubbled with 95% O2 and 5% CO2, and contained Krebs-Henseleit solution (in mmol/l: 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.4 KH2PO4, 25 NaHCO3, and 11 glucose).

Electrical stimulation via punctate platinum bipolar electrodes with rectangular pulses (1-ms duration) at an intensity of 50% above threshold was delivered at intervals of 2,000 ms (14). The effect of EG (range 5 × 10−5–10−8 mol/l) on inhibition of Lzm-S-induced (1 × 10−6 mol/l) myocardial depression was examined at 10 min and 20 min after instillation. Isometric contraction measured in grams was converted into units of tension (mN/mm2) in which grams per cross-sectional area was multiplied by the acceleration due to gravity, 9.80 cm/s². Cross-sectional area was determined as muscle weight/specific gravity (1.06 g/ml/muscle length) (24).

In Vivo Study

E. coli model and animal preparation. The E. coli model used in these studies was identical to that previously described (14, 22, 26, 30). Sepsis was induced by the intravenous infusion of 1010 colony-forming units of live E. coli (designation 0111:B4). The bacteria were suspended in normal saline solution and were given over 0.5 h. A constant infusion of ~5 × 108 colony-forming units/h of E. coli was then maintained over the duration of the study.

After induction with thiopental sodium (20 mg/kg iv), the animals (16–27 kg) were constantly infused with sufentanil citrate (0.05–0.3 μg/min) and midazolam (5 μg·kg−1·min−1) (26). Drug rates were adjusted as required to abolish the palpebral reflex. After tracheal intubation, the animals were mechanically ventilated (tidal volume of 20 ml/kg) in the supine position. The ventilator rate was initially set to maintain blood pH at ~7.35 and was increased as necessary to reverse the metabolic acidosis that developed over the course of the study. Approximately 100% oxygen was inspired, so that hypoxemia would not affect the study.

Under sterile conditions, a thermistor-tipped catheter was advanced through a percutaneously inserted introducer (Arrow International, Reading, PA) from the right jugular vein into the pulmonary artery to measure mean pulmonary arterial pressure (Ppa), mean pulmonary capillary wedge pressure (Ppw), mean right atrial pressure (RAP), and thermodilution cardiac output (CO) (Edwards Lifesciences, Irvine, CA). A similar introducer and a triple lumen catheter were positioned into the left jugular vein for administration of intravenous saline solution, the E. coli infusion, and the treatment or placebo drugs (see below). Urine output was determined by a catheter placed into the bladder by way of the urethra. Another polyethylene catheter was percutaneously placed into the femoral artery to measure mean arterial blood pressure (MAP) and to obtain samples of blood for arterial PO2, PCO2, pH, hematocrit, serum electrolytes and creatinine, liver function tests, lactate concentrations, and other measurements (see below). The clinical laboratory tests were performed by the chemistry laboratories at the Health Sciences Centre.

All vascular catheters were connected to pressure transducers (Cobe, Argon Medical Devices, Athens, TX), were referenced relative to the left atrium, and were connected to a chart recorder (Astro-Med, West Warwick, RI). Heart rate (HR) was measured from the recorder tracing. Stroke volume (SV) was calculated as CO/HR. Systemic vascular resistance (SVR) was calculated from [(MAP − RAP)/CO] × 80. MAP was our primary index of cardiac performance and the primary hemodynamic end point chosen. Hemodynamics were determined at a constant Ppw over the course of the study. The baseline Ppw usually averaged ~8–10 mmHg, and normal saline solution was given as necessary to maintain Ppw constant over the experimental period. Stroke work (SW) was calculated from SV × (MAP − Ppw). Experimental procedure. The primary objective was to determine whether EG could reverse the fall in MAP once this occurred in this sepsis bacteremic model. EG was initiated after MAP pressure fell ~40% from the baseline value. This decline in MAP was not responsive to volume infusion (i.e., normal saline solution) to return Ppw to the baseline value. Four groups were examined, which included two septic groups and two nonseptic groups. In the two septic groups, the animals were randomized to receive EG and volume infusion or placebo treatment and volume infusion. Normal saline solution was
the diluent for EG and was given as the placebo treatment. In the two nonseptic groups, one received EG while the other received placebo treatment. In the nonseptic groups, only maintenance volume for anesthesia and for placebo drugs was given over the course of the experiment.

EG was infused with the goal of achieving plasma concentrations in the $10^{-5}$ mol/l range based on our previous work in this bacteremic model (30) (see DISCUSSION). EG was administered as a solution in which 2 g were dissolved in 250 ml of normal saline solution. On initiation, 80 mg/kg EG was infused over an approximate 1-h period, after which the rate was turned down to 40 mg·kg$^{-1}$·h$^{-1}$ and could be subsequently titrated further downward as long as MAP remained at the baseline value. Hemodynamic measurements were obtained at baseline, once septic shock had been obtained, and at 1 h, 2 h, 3 h, 4 h, and 5 h after treatment. In the nonseptic groups, the sham shock condition represents the mean time for the development of shock found in the septic groups ($\sim$5 h). In both groups, in addition to hemodynamic measurements, blood chemistries and arterial blood gas measurements were obtained at baseline, in the septic/sham shock condition, and at 3 h and 5 h after treatment. The animals were killed after completion of the studies.

Serum EG concentrations were determined by liquid chromatography (LC)-mass spectroscopy (MS) techniques by Matrix BioAnalytical Laboratories (Woodbridge, CT). The analyte was extracted from plasma by protein precipitation. Fifty microfilters of blank matrix calibration standards, quality controls, and study samples were pipetted into a 96-well plate. One hundred fifty microfilters of acetonitrile-containing internal standard was added to all samples to facilitate protein precipitation. The plate was vortex-mixed for 3 min and then allowed to stand for at least 30 min in the refrigerator (2–8°C). Samples were then centrifuged for 10 min. The supernatant (130 µl) from each sample was then transferred to a new 96-well plate, and 130 µl of mobile phase A (95% H$_2$O, 5% CH$_3$CN, 0.1% formic acid) was added to the supernatant and vortex-mixed for 3 min. Subsequently, the solutions were centrifuged for 10 min, and 20 µl of each supernatant was injected into the HPLC-tandem MS system for quantification (analytical column, SymmetryShield RP8 3.5 µm, 2.1 × 50 mm; Waters 1525 Binary HPLC Pump at 0.35 ml/min; Quattro UltimaTM Tandem MS/MS, Waters Micromass). The standard curves were calculated from the ratios of analyte/internal standard for EG of the calibration standards and their nominal analyte concentrations.

The unknown samples were calculated from quadratic regression where a weighted curve, 1/x, was used. Data were recorded with MassLynx version 4.1, and all integration was performed with MassLynx version 4.1. The concentration range detected in the canine serum demonstrated a response function with a correlation coefficient ($r^2$) over 0.99.

Serum samples from each of the four groups were also analyzed for H$_2$O$_2$ concentrations at the baseline, in the septic/sham shock condition, and at 3 and 5 h after treatment by a fluorometric assay as described further below. Furthermore, since EG might have altered oxidant stress related to NO formation and metabolism, serum measurements of total nitrite (after conversion of nitrate to nitrite by oxidant stress related to NO formation and metabolism, serum measurements of total nitrate (after conversion of nitrate to nitrite by nitrate reductase) were compared by a colorimetric assay between the preparations. Then, in a subsequent experiment performed in a model system, it was determined whether EG could also eliminate the generation of H$_2$O$_2$ (38).

To Determine Whether Ethyl Gallate Scavenges H$_2$O$_2$

The Ultra Amplex Red fluorometric hydrogen peroxide assay (Molecular Probes, Eugene, OR) was first used to measure H$_2$O$_2$ concentrations to determine EG’s scavenging ability in model systems and in the in vivo preparation (12, 24). In an initial experiment in which a model system was used, $10^{-5}$ mol/l H$_2$O$_2$ was placed into respective solutions that contained EG (range $10^{-5}$–$10^{-3}$ mol/l) for $\sim$20 min, after which the samples were frozen and subsequently analyzed for H$_2$O$_2$ to see whether EG reduced the H$_2$O$_2$ concentrations in this preparation. Then, in a subsequent experiment performed in a model system, it was determined whether EG could also eliminate the generation of H$_2$O$_2$ caused by Lzm-S. As previously described, Lzm-S (2 × $10^{-5}$ mol/l) was placed into a 2-ml vial, filled to the $\sim$1-ml mark with Hanks’ balanced salt solution, and studied at 35°C and pH 7.35 (24). After $\sim$20 min of exposure to visible light, the sample was frozen at $-20^\circ$C and was later studied for H$_2$O$_2$ generation by this fluorose assay technique.

In the in vivo experiments, the Ultra Amplex Red fluorometric hydrogen peroxide assay was also used to detect H$_2$O$_2$ blood concentrations in which the blood samples were obtained at baseline, in septic/sham shock, and at 3 h and 5 h posttreatment conditions. For these measurements, after the blood sample was drawn from the venous catheter it was rapidly centrifuged and the red blood cells separated from the serum. The sample was quickly stored at $-20^\circ$C, and then the fluorose analysis was performed. In all of these Ultra Amplex Red assay experiments, moreover, any background fluorescent activity attributable to EG was subtracted from the total amount in the interpretation of these results.

Besides the fluorometric H$_2$O$_2$ technique, it was additionally determined whether EG could scavenge H$_2$O$_2$ in model systems in which the electrochemical probe technique was used as described previously (HPO-100 Sensor, Apollo 400 Radical Analyzer, Precision Instruments, Sarasota, FL) (24). In an initial set of experiments, stock solutions of H$_2$O$_2$ ($10^{-5}$ mol/l) were placed into respective vials that contained EG (range $1 \times 10^{-4}$–$1 \times 10^{-5}$ mol/l). Then, after the addition of H$_2$O$_2$, the change in H$_2$O$_2$ was followed over a 20-min interval to determine whether EG could reduce the amount of H$_2$O$_2$ detected by the probe. Afterwards, in a second experiment it was determined whether EG could slow the generation of H$_2$O$_2$ when a glucose oxidase/glucose system was used for H$_2$O$_2$ generation as determined by the electrochemical probe technique. Glucose oxidase (obtained from Aspergillus niger, 1 and 2.5 U, respectively; Sigma) was added to a 10-ml vial of distilled water (pH $\sim$7.00) that contained glucose (2.7 × $10^{-3}$ mol/l). The resultant generation of H$_2$O$_2$ was monitored over a 10-min period with and without EG pretreatment ($5 \times 10^{-4}$ mol/l). The effect of bovine liver catalase (1.3 × $10^{-6}$ mol/l) on inhibiting the H$_2$O$_2$ signal generated by glucose oxidase/glucose preparation was also determined as part of this experimental protocol.

To Determine by Spectroscopy Techniques Whether Ethyl Gallate Preserves Native Catalase in Degradation of H$_2$O$_2$

The ability of EG to prevent the conversion of catalase by peroxoacetic acid, an H$_2$O$_2$ analog, was studied by spectroscopy techniques (7, 8, 11, 34). Investigators have shown that acyl-hydroperoxides (peroxygen acids) provide suitable catalase pseudosubstrates, and it is therefore possible to form a stable compound I by the reaction of peroxygen acid and catalase (7, 8, 20). Compound I is a derivative of native catalase and is the oxidized form of the native enzyme. In previous experiments, this compound was shown to be an important contributor to both Lzm-S-induced vasodilation and myocardial depression in in vitro preparations (24, 27). Phenols can reduce compound I to compound II and then reduce compound II back to the native enzyme in one electron step (34). By spectroscopy techniques, catalase has a characteristic peak absorbance at 405 nm (11, 34), and it was determined whether EG could prevent the degradation of this

**J Appl Physiol • VOL 110 • FEBRUARY 2011 • www.jap.org**
enzyme in the present study. Peroxoaacetic acid was added to a bovine catalase solution (1.3 × 10⁻⁶ mol/l, pH 7.4; Sigma), and the subsequent change in absorbance was followed for a duration of 15 min by wave-scan spectroscopy techniques (Pharmacia Biotech, Ultraspec 3000). Measurements were obtained at baseline, immediately after addition of peroxoaacetic acid (4.5 × 10⁻⁻⁷ mol/l), and at 5, 10, and 15 min thereafter with and without EG (10⁻³ mol/l) treatment.

Statistical Analyses

In our analyses, the results obtained for a given inhibitor concentration in the organ bath preparation or treatment in the dog study were compared by two-way ANOVA in which there are different groups (i.e., one level of comparison) and different time periods (second level of comparison) (GB-Stat V8, Dynamic Microsystems, Silver Spring, MD). In addition, a one-way randomized ANOVA was used for statistical analysis of the H₂O₂ concentrations in model systems (see Fig. 7). From the results of the ANOVA tables, a Student-Newman-Keuls comparison test was used to determine where differences were observed among groups at a specific time period. Differences in intravenous fluid volume between septic groups were compared by unpaired t-test. Results are reported as means ± SD.

RESULTS

Of the compounds tested, the results showed that EG, methyl gallate, and ethyl 3,4-dihydroxybenzoate prevented Lzm-S-induced vasodilation in the phenylephrine-constricted carotid artery preparation (see Figs. 1 and 2). In Fig. 1A, it is shown that Lzm-S alone caused a reduction in force to ~50% of the baseline value. In previous studies, this reduction in force could not be observed with denatured Lzm-S or with α-lactalbumin, a substance that is closely related to Lzm-S (24, 27). In Fig. 1B, the results are plotted as percentage of the baseline value. In the range of 10⁻⁴–10⁻⁵ mol/l, EG prevented vasodilation caused by Lzm-S in a dose-response manner. By itself, EG did not have a significant effect on force compared with the buffer-treated time control group.

In the ventricular trabecular preparation, Lzm-S caused a decrease in tension of ~50% compared with baseline. EG concentrations as low as 10⁻⁷ mol/l were able to lessen this depression (see Fig. 3).

In Vivo Study

Sepsis groups. In the two sepsis groups, baseline hemodynamic variables (see below) were not different between the EG-treated and non-EG-treated groups. None of the dogs died over the course of the study. In the EG-treated group the average time to initiation of treatment after E. coli infusion was 251 ± 140 min, while in the placebo-treated group the average time to initiation of normal saline solution (placebo treatment) was 313 ± 68 min after baseline. In two animals, blood cultures were obtained in the EG-treated group in which E. coli was grown from the respective samples.

An example obtained in the EG-treated sepsis group is shown in Fig. 4A, in which MAP decreased ~50% from the baseline (presepsis) value. EG infusion caused a sustained increase in MAP that was evident by 20 min after infusion. The mean values of MAP are shown in Fig. 4C, where it can be seen that in the septic condition MAP dropped ~40% from the baseline value and that EG restored MAP to the baseline value for the duration of the study. After the initial dose of EG (i.e., 80 mg/kg), EG could be titrated downward somewhat to keep MAP at the baseline value. The average total dose of EG given in the septic group over the 5-h period was 0.186 ± 0.02 g/kg with a range between 0.15 and 0.22 g/kg. Furthermore, in the placebo-treated group, MAP also decreased ~40% from the baseline value, where it remained over the course of the study.

In both septic groups, SVR in the septic shock condition (i.e., ~5 h after E. coli was infused) decreased compared with the baseline condition. In the EG-treated group, the effect of EG on MAP was to cause an increase in SVR (see Fig. 4D). In this group, SVR significantly increased after treatment, both compared with the shock condition and compared with the corresponding values found in the nontreated septic group. Despite the increase in SVR caused by EG, which in turn would be expected to increase left ventricular afterload (31), CO and SV did not fall relatively in the EG-treated group. Indeed, when the changes in SW from baseline to the different
The findings for Ppa and RAP over the course of the study were not different between the two groups. RAP remained at ~9 mmHg over the different intervals of the study. In the treated group, Ppa increased from 16 ± 1.3 mmHg to 23 ± 7 mmHg (P < 0.05 vs. baseline) at the end of the study, while in the nontreated group Ppa increased from 18 ± 2.5 mmHg at baseline to 20 ± 4 mmHg at the end of the study. In both groups, HR increased to similar extents over the course of the study. In the treated group, HR averaged 59 ± 8 beats/min at baseline, peaked to 144 ± 33 beats/min (P < 0.05 vs. baseline) at the septic shock condition, and subsequently decreased to 110 ± 34 beats/min at 5 h after treatment (P < 0.05 vs. baseline). In the nontreated group, HR measured 56 ± 6 beats/min at baseline, peaked to 137 ± 25 beats/min (P < 0.05 vs. baseline) at the septic shock condition, and subsequently decreased to 128 ± 28 beats/min at 5 h after treatment (P < 0.05 vs. baseline). Furthermore, over the period of sepsis, the ventilatory rate was increased in order to reverse the non-ion and ion-gap metabolic acidosis that develops in this model. In both groups, pH and arterial PCO2 fell to similar extents from baseline at the different measurement intervals. In the treated group respective values of pH and PCO2 decreased from 7.38 ± 0.03 and 29 ± 4 mmHg at baseline to 7.14 ± 0.06 and 19 ± 2 mmHg at the end of the study (both P < 0.05 from baseline), while in the nontreated group the corresponding values were from 7.41 ± 0.03 and 31 ± 5 mmHg at baseline to 7.11 ± 0.07 and 22 ± 4 mmHg at the end of the study (both P < 0.05 from baseline). Arterial lactate was significantly increased compared with baseline in the nontreated EG septic group, but the results were not different between the two groups (see Fig. 6A).

According to the overall design of this study, intravenous volume was administered in both groups to maintain Ppw at the baseline value throughout the study. Significantly less normal saline was required in the EG-treated group to achieve this goal (3.38 ± 0.81 l vs. 4.7 ± 0.81 l; P < 0.05). At the end of the study, serum hemoglobin concentration in the EG-treated septic group was higher compared with the non-EG-treated septic group (see Fig. 6C). In addition, in the EG-treated septic group, serum creatinine, the primary index of renal function, decreased over the course of the study, which indicated improved renal function, while it increased in the nontreated septic group (see Fig. 6B). Urine output in the EG-treated septic group was greater (~40 vs. 15 ml/h) over the last 2 h than in the nontreated septic group but did not reach statistical significance between the two groups. Because of rhabdomyolysis, serum creatine kinase (CK) increases in this model (26). This effect appeared greater in the nontreated septic group (see Fig. 6D). There was no evidence to suggest that EG affected liver function or hematological parameters in this study (see Table 1).

Nonseptic groups. In the EG-treated group, the average dose of EG given was 0.26 ± 0.02 g/kg with a range of 0.22–0.28 g/kg (P < 0.01 vs. septic group). The total amount of EG given in the nonseptic group was higher than that in the septic group. The reason was because the EG infusion was not reduced over the course of the study in the nonseptic group, since there were no changes in hemodynamics that lent themselves to titrating a rate change. EG was thus maintained at the ~40 mg·kg⁻¹·h⁻¹ rate after the initial 80 mg/kg infusion that was given over the first hour.

In contrast to the septic animal, EG treatment did not cause a change in MAP over this period. An example is shown in Fig. 4B, while the mean data are shown in Fig. 4C. Indeed, the results indicate that, if anything, EG treatment caused a decrease in SVR compared with pretreatment in the nonseptic group (Fig. 4D; see DISCUSSION). Moreover, at the end of the study, CO, SV, and SW were increased with EG infusion in the nonseptic animal (see Fig. 5) compared with the volume-infused time control group. In the EG-infused group, creatinine significantly decreased over the course of the study compared with the nontreated group (see Fig. 6). In addition, HR increased with EG treatment at the end of the study (71 ± 10 vs. 45 ± 6 beats/min, P < .05 between groups; see DISCUSSION), while Ppa, RAP, Pco2, arterial pH, blood chemistry and hematological values, and urine volume did not appreciably change.
over the course of the study and were not significantly different between the two groups (data not shown).

EG serum concentrations were measured in the treated septic and nonseptic groups by LC-MS techniques. In one animal in each group, there was a very small amount of EG detected in the serum before EG administration, although it is not clear how the animal’s blood came in contact with the substance. There were no differences in EG concentrations between the septic and nonseptic groups. In the septic group, EG measured 1.8 \pm 0.5 \times 10^{-6} \text{mol/l} at 5 h after treatment. In the nonseptic group, the values were 2.7 \pm 10^{-6} \text{mol/l} at baseline (due to 1 dog), 0 at the sham shock condition, 2.85 \pm 1.27 \times 10^{-6} \text{mol/l} at 3 h after treatment, and 2.8 \pm .42 \times 10^{-6} \text{mol/l} at 5 h after treatment.

**Ethyl Gallate Scavenges H_2O_2**

As detected by the Ultra Amplex Red assay, EG markedly attenuated the concentration of H_2O_2 when generated by Lzm-S as well as when H_2O_2 was exogenously administered. In the experiment in which Lzm-S was found to intrinsically generate 2.4 \times 10^{-6} \text{mol/l} of H_2O_2 over the 20-min interval in a model system, no H_2O_2 could be detected when EG (10^{-4} \text{mol/l}) was added. In another model system, moreover, when 10^{-5} \text{mol/l} H_2O_2 was added to concentrations of EG (10^{-3}–10^{-5} \text{mol/l}), the amount of H_2O_2 detected fell to \sim 10–20% of the nontreated value (see Fig. 7A).

In the electrochemical probe experiment, the same electrochemical probe was used in the EG-treated and nontreated conditions, since the sensitivity of the probe to the exogenously administered H_2O_2 differed among the various probes. In Fig. 7B, it can be seen that the peak amplitude of the H_2O_2 signal...
was much less in the EG-treated condition. In addition, at the $10^{-4}$ and $5 \times 10^{-5}$ mol/l EG concentrations, it was particularly observed that the signal declined over the 20-min measurement period, as $H_2O_2$ was continually degraded over this interval. At $10^{-4}$ mol/l EG, the initial signal and that measured at 20 min after $H_2O_2$ instillation were 49$\pm$12% and 15$\pm$18% ($n=6$), respectively, of the values found for the nontreated sample. At $5 \times 10^{-5}$ mol/l EG, the corresponding values were 58$\pm$18% and 49$\pm$13% ($n=5$). At $10^{-5}$ mol/l EG, the corresponding values were 78$\pm$14% and 67$\pm$14% ($n=6$, $P<0.05$ treated vs. nontreated preparations for all EG treatments). Furthermore, any differences in the percent declines in $H_2O_2$ observed between the Ultra Amplex Red assay and the probe methodologies probably reflected the length of time that $H_2O_2$ remained in contact with EG, since the determinations for the Ultra Amplex Red assay measurements were only performed when enough samples had been obtained, usually 2–3 days after the reaction was initiated.

The effect of EG ($5 \times 10^{-4}$ mol/l) on $H_2O_2$ generation was also determined in a glucose oxidase/glucose model system in which this enzyme system caused a constant rate of $H_2O_2$ production. As determined by the probe (see Fig. 7C), the $y$-axis represents the signal proportional to the amount of $H_2O_2$ generated, which is given in picoamperes. As shown in Fig. 7C, there was a dose-response effect of glucose oxidase on $H_2O_2$ production in which the 2.5 U dosage of glucose oxidase produced about two times the rate of $H_2O_2$ generation as that found with the 1 U value. For the 2.5 U dosage, the mean ($\pm$SD) rate of $H_2O_2$ generation was $7.8 \pm 0.9 \times 10^{-5}$ mol $H_2O_2$·l$^{-1}$·min$^{-1}$ in the untreated sample ($n=6$) and decreased to $5.7 \pm 1.9 \times 10^{-5}$ mol $H_2O_2$·l$^{-1}$·min$^{-1}$ with EG treatment ($P<0.05$ vs. untreated). For this high glucose...
oxidase concentration, EG caused a mean reduction in the rate of H$_2$O$_2$ generated that averaged an ~30% decrease of the nontreated condition. For the 1 U glucose oxidase concentration, the mean (SD) H$_2$O$_2$ generation in the untreated condition was $3.2 \pm 1 \times 10^{-5}$ mol H$_2$O$_2$·l$^{-1}$·min$^{-1}$ ($n = 6$) and decreased to $1.4 \pm 0.6 \times 10^{-5}$ mol H$_2$O$_2$·l$^{-1}$·min$^{-1}$ with treatment ($P < 0.05$ vs. untreated). For this low glucose oxidase concentration, EG caused a mean H$_2$O$_2$ reduction that averaged an ~30% decrease of the nontreated condition. In the presence of bovine liver catalase, the production of H$_2$O$_2$ by 1 U of glucose oxidase was completely extinguished ($0.15 \pm 0.09 \times 10^{-5}$ mol H$_2$O$_2$·l$^{-1}$·min$^{-1}$; $n = 4$). In the presence of EG (5 × 10$^{-4}$ mol/l), moreover, it was also shown that instillation of placebo (i.e., distilled water) caused no change in the signal ($-0.25 \pm 0.4 \times 10^{-5}$ mol H$_2$O$_2$·l$^{-1}$·min$^{-1}$; $n = 4$). Furthermore, no production of H$_2$O$_2$ was detected when glucose oxidase was added to distilled water that contained no glucose.

As part of the in vivo canine sepsis study, serum H$_2$O$_2$ concentrations were measured by Ultra Amplex Red assay at baseline (presepsis), in the shock/sham shock condition, and at 3 h and 5 h after the septic shock condition. The results showed that in the non-EG-treated septic shock group there were small but significant increases in H$_2$O$_2$ detected over the course of the study, particularly at the last measurement reading. In this group, the results measured $0.36 \pm 0.05 \times 10^{-7}$ mol/l at baseline, $0.36 \pm 0.05 \times 10^{-7}$ mol/l at the septic shock condition, $0.49 \pm 0.03 \times 10^{-7}$ mol/l at 3 h after the septic shock condition, and $0.78 \pm 0.45 \times 10^{-7}$ mol/l ($P < 0.05$ vs. baseline) at 5 h after the septic shock condition. In the non-septic time control group, however, there were no changes over time, and the corresponding results were $0.47 \pm 0.08 \times 10^{-7}$ mol/l, $0.42 \pm 0.05 \times 10^{-7}$ mol/l, $0.40 \pm 0.39 \times 10^{-7}$ mol/l, and $0.46 \pm 0.14 \times 10^{-7}$ mol/l, respectively. The relative increase in H$_2$O$_2$ from the baseline to the 5 h posttreatment condition was significantly different between the two groups.

**Fig. 5.** Measurements of cardiac performance in the septic and nonseptic groups. In the septic group, despite the increase in SVR mediated by EG infusion, cardiac output (A) and stroke volume (B) did not fall compared with the nontreated group. For similar pulmonary wedge pressures between groups (D), the fall in stroke work (C) from baseline over the course of the study was less in the EG-treated group. In the nontreated group, EG caused an increase in cardiac output (A), stroke volume (B), and stroke work (C) for similar pulmonary wedge pressures (D) between groups. Statistical analyses included 2-way repeated-measures ANOVA and SNK multiple comparison test: *$P < 0.05$ vs. baseline; †$P < 0.05$ vs. all other measurements in a group; ‡$P < 0.05$ between respective groups; §$P < 0.05$ between groups in which the change in stroke work from baseline was used in the analysis.
On the other hand, in the EG-treated septic and nonseptic groups EG produced some background fluorescence in the sample, but there was no difference in fluorescent signals between these two groups over the course of the study.

In terms of the NO determinations, EG infusion did not appear to modify NO formation/metabolism in the septic group as determined by serum total nitrite and 3-nitrotyrosine concentrations. For the nitrite analysis, serum concentrations increased to similar extents in both groups. In the nontreated septic group, the mean values of nitrite at the three conditions (baseline, septic shock, and 5 h after treatment) were 2.08 ± 0.746, 2.64 ± 0.52, and 3.34 ± 1.3 (P < 0.05 vs. baseline) respectively, while in the EG-treated septic group, the corresponding values were 1.877 ± 1.12, 2.64 ± 0.8, and 3.81 ± 1.16 (P < 0.05 vs. baseline) μM, respectively. For the 3-nitrotyrosine analysis, there was a large variation in baseline values among the animals that ranged from 10 to 131 nM in the two groups, so that the individual changes in nitrotyrosine serum concentrations over the three conditions were expressed as a percentage of the respective baseline values. In the nontreated septic group, the mean nitrotyrosine concentration increased to 4 ± 10 times the baseline value at the septic shock condition and then decreased to 2.2 ± 3.4 times the baseline value at the end of the experiment. In the EG-treated septic group, the mean nitrotyrosine concentration increased to 5 ± 11 times the baseline value at the septic shock condition, and then decreased to 3.9 ± 8 times the baseline value at the end of the experiment. However, no significant changes were observed between groups or among the different conditions studied in this model. In addition, there were no differences between the two groups when the data were analyzed in a different manner, for instance, in terms of the absolute values.

Ethyl Gallate Limits Formation of Catalase-Related Derivatives

Wave-scan spectroscopy was used to assess whether after peroxyacetic acid administration EG could preserve and limit the conversion of native catalase to its derivatives. In Fig. 8, A and B, examples are shown without and with EG treatment, respectively. In this figure, an absorption peak at 405 nm can be observed, which represents the Soret peak of catalase (10). When peroxyacetic acid (4.5 × 10⁻⁵ mol/l) was added to catalase, there was a decline in this peak as catalase was converted to its derivatives. Measurements were determined immediately after and at 5, 10, and 15 min after instillation of
In the presence of EG ($10^{-3}$ mol/l), the decline in the catalase peak was attenuated compared with the nontreated preparation. On the mean, the absorbance at baseline was not different between the EG-treated ($n=6$) and nontreated groups ($n=6$; 0.48 ± 0.057 vs. 0.48 ± 0.039). However, the change in absorbance after addition of peroxoacetic acid was significantly less over the four conditions in the EG-treated group (see Fig. 8C).

### DISCUSSION

Based on the recent discovery that Lzm-S could intrinsically generate H$_2$O$_2$ (24) by a mechanism consistent with that described by Wentworth and colleagues (10, 35, 43–46), we used our carotid artery preparation as a bioassay and looked for compounds that were antioxidants with the capacity to inhibit LzM-S-induced vasodilation. As determined by previous experiments, Lzm-S’s ability to cause cardiovascular dysfunction in the in vitro preparations appears solely due to the generation of H$_2$O$_2$, since we could not demonstrate that reactive oxygen species other than H$_2$O$_2$ participated in Lzm-S-induced vasodilation or myocardial depression in these preparations (24, 27). In the present study, we found that of the antioxidants examined EG was the most favorable in the inhibition of Lzm-S-induced effects in the in vitro preparations. EG is added to food as an antioxidant (17, 32, 47) and is the ethyl ester of gallic acid. EG is found naturally occurring in a variety of plant sources including red wine and walnuts. In the laboratory, EG can be produced from gallic acid and ethanol (42). The chemical structure of EG is shown in Fig. 9A.

Once we found that EG could inhibit Lzm-S’s vasodilatory effect in the carotid organ bath preparation, we then tested whether this treatment would be beneficial in reversing the hypotension that develops in our canine model. We found that there were many beneficial effects of EG treatment on end-organ function and hemodynamics in this model. In the treated septic group, the results showed not only that EG was very effective in reversing the hypotension that develops in this model but also that this occurred within 20 min of administration and that MAP could be maintained comparable to the baseline value over the entire course of the experiment. The mechanism of this increase in MAP was due to an increase in SVR. As shown in Fig. 4D, SVR decreased in the septic shock condition compared with baseline and increased with EG treatment. These findings would be consistent with those found in the carotid artery organ bath preparation, where EG also prevented the vasodilation caused by Lzm-S. In the septic group, despite the increase in left ventricular afterload that would otherwise lead to a decrease in SV in this model (31), cardiac performance additionally appeared better in the EG-treated group compared with the nontreated group, also consistent with the results obtained in the isolated RVT preparation. In addition, in the EG-treated group oxygen delivery trended higher compared with the nontreated group ($154 \pm 67$ vs. $123 \pm 56$ ml oxygen/min), but our study was not powered to examine this question.

It is also noteworthy that we found that the beneficial effects of EG on myocardial function in the septic group were somewhat less than that found for the SVR measurements. There are a few explanations for this observation. First, mediators other than Lzm-S could have been released in this model that were not amenable to EG treatment (1, 36). Second, there were serum electrolyte and acid-base disturbances that evolved over the course of the study that may have additionally contributed to the myocardial dysfunction observed. Because of difficulties in obtaining immediate laboratory results, we did not include replacement of electrolytes or correction of acid/base disturbances as part of the protocol. All of the aforementioned reasons could have limited the ability of EG to improve myocardial function in the in vivo study.

Besides its overall beneficial effect on hemodynamics, EG also appeared to improve end-organ function in septic shock. At the conclusion of the in vivo study, serum creatinine was less in the EG-treated group than in the nontreated septic group, a finding indicating that renal function was better preserved in the treated group. Hsieh et al. (18) showed that methyl gallate protected against H$_2$O$_2$-induced oxidative stress...
Fig. 7. EG scavenges hydrogen peroxide (H$_2$O$_2$). In A, stock solutions of H$_2$O$_2$ were placed into respective vials that contained different concentrations of EG. By Ultra Amplex Red assay, there was less H$_2$O$_2$ detected in the EG-treated solutions after 20 min of incubation for an identical H$_2$O$_2$ concentration instilled in each condition. Statistical analyses included a 1-way randomized ANOVA and SNK multiple comparison test: *$P < 0.05$ vs. all other groups; †$P < 0.05$ vs. H$_2$O$_2$ alone and H$_2$O$_2$ and EG 10$^{-3}$ mol/l groups. In B, H$_2$O$_2$ concentrations were measured by the electrochemical probe technique. In the untreated condition, instillation of H$_2$O$_2$ (10$^{-5}$ mol/l) produced a constant signal over the duration of the experiment. In the EG treatment experiment, for the same amount of H$_2$O$_2$ the signal peak was attenuated and declined over time. This was particularly evident at the 2 highest EG concentrations. In C, H$_2$O$_2$ was generated by a glucose oxidase/glucose (GO) preparation in which measurements were obtained by the electrochemical probe. The $y$-axis represents the electrochemical probe signal, which is proportional to the amount of H$_2$O$_2$ generated and which is measured in picoamperes (pA). As shown in a and b, there was a dose-response effect of GO on H$_2$O$_2$ production. EG slowed the generation of H$_2$O$_2$ at each concentration of GO used. Pretreatment (c) or posttreatment (d) of the solution with catalase eliminated the detection of H$_2$O$_2$ in this model system; see text for discussion.
and DNA damage in canine kidney cells, and EG could perform a similar function in sepsis. In the treated group, less intravenous fluid was also required to maintain Ppw at the baseline value, so that hemoglobin did not decrease as much in this group. We previously used serum CK as a marker of rhabdomyolysis in this model (26). In the present study, we found that CK was significantly reduced at the end of the study in the EG group compared with the nontreated group, so that it appeared that muscle injury occurred to a lesser extent in the treated group. All these factors point to better end-organ functioning in addition to better hemodynamic performance with EG treatment in this septic shock model.

In the nonseptic group, EG also had significant effects on hemodynamics, but these effects were somewhat different from those observed in the septic group. In the nonseptic group, MAP and SVR did not increase with EG treatment, so that EG does not act as a nonspecific vasoconstrictor and does not increase SVR under nonseptic conditions. We previously showed (25) that Lzm-S causes a reduction in the cardiac sympathetic response in an in vitro RVT preparation and that this occurs by NO signaling. We also know that there is some Lzm-S that can be detected in the blood of nonseptic dogs (29). We think, therefore, that there might be some cardiac H2O2 signaling going on even in the nonseptic animal and that EG caused an inhibition of this signaling leading to an increase in HR and CO and therefore to reflex vasodilation in this nonseptic group. In addition, we found that EG also improved renal function as indicated by the fact that creatinine decreased over the course of the experiment in this group. This means that there may also be H2O2-dependent pathways present that modulate renal function under nonseptic conditions.

In the untreated control group, we also noted that there was an increase in SVR over time compared with the baseline measurement (see Fig. 4D). This finding was not related to any

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**Fig. 8.** EG limits formation of catalase-related derivatives. Wave-scan spectroscopy techniques were used to examine the effect of peroxoacetic acid on the conversion of catalase to its derivatives as identified by the decline in the peak at 405 nm. In A, when peroxoacetic acid was added to the solution, there was a marked decline in absorbance at 405 nm as catalase was converted to its derivative compounds. In B, EG attenuated the decline in the catalase peak and preserved native catalase. In C, the mean data are shown in which change in absorbance at 405 nm was determined. The decrease in native catalase in the EG-treated group was less than that found in the nontreated group. Statistical analyses included 2-way repeated-measures ANOVA and SNK multiple comparison test: *P < 0.05 between groups.
change in volume status, since we kept our measurement of preload relatively constant over the duration of the experiment. Rather, we think that this increase in SVR was due to the fact that the animals develop a significant bradycardia after many hours of sufentanil-midazolam anesthesia in this preparation. As a result, there was a tendency for CO to fall at the later measurement intervals (see Fig. 5A). In turn, this fall resulted in a reflex vasoconstriction and hence an increase in SVR in the untreated time control group. On the other hand, in the non-treated and EG-treated septic groups, although CO also decreased over time this effect was more likely primarily related to the myocardial depression and the metabolic derangements that occurred (as mentioned above), rather than to the anesthesia used. In these groups, such reductions in CO would also lead to relative increases in SVR by reflex vasoconstriction, as was detected at the later measurement intervals. In the non-treated septic group, we found that SVR decreased to the lowest extent in the septic shock condition (see Fig. 4D) and that SVR then relatively increased by this reflex vasoconstriction at the later measurement periods. Because of these slight increases in SVR that occurred over time in the nontreated septic group, we found them comparable between the treated and nontreated septic groups.

The pathway by which Lzm-S causes vasodilation in vitro is shown in Fig. 9B. As previously discussed, we believe that H2O2 is generated by the mechanism described by Wentworth and colleagues (43–46), after which it diffuses across the membrane to be metabolized by catalase. We have not been able to identify a receptor to which H2O2 binds (27). In Fig. 9, we have also delineated the substances previously described to inhibit and/or potentiate the pathway of Lzm-S-induced vasodilation (27). As shown, ethanol, which inhibits the formation of compound I, is also included in this figure, since its discovery was pivotal to our understanding that H2O2 degradation to compound I was an important component to Lzm-S’s mechanism of action (27). In the present study, we further show that one mechanism by which EG can inhibit Lzm-S’s vasodilatory effect is by scavenging H2O2, as we demonstrated by fluorometric and electrochemical probe techniques. In Fig. 7Cd, we also show that after catalase was added to the system there was a precipitous fall in H2O2, since catalase is such an effective scavenger of H2O2 that occurs at a rate of ~5 mmol/s (34). Hall et al. (17) similarly demonstrated that EG could scavenge H2O2. In the presence of H2O2, these investigators found that the ethyl hydroxybenzoates decompose with the ultimate formation of dihydroxybenzenes. In addition to scavenging H2O2, our spectroscopic findings also indicate that during the reaction with hydroperoxides, EG can limit the formation of catalase-related derivatives, and found them comparable between the treated and nontreated septic groups.

**Fig. 9.** A: structure of the EG molecule. B: mechanism of lysozyme-induced vasodilation. Lysozyme generates H2O2, which is metabolized by catalase to form its derivatives, such as compound I. Compound I leads to an increase in cyclic guanosine monophosphate (cGMP) to produce vasodilation. Aspergillus niger catalase (which would metabolize H2O2) and ethanol (an inhibitor of formation of compound I; Ref. 27) can prevent lysozyme’s effects. Peroxooxidic acid can generate compound I (20, 24). EG can scavenge H2O2 and can also attenuate the formation of catalase-related derivatives.
action that would reduce the availability of compound I in contributing to Lzm-S-induced effects. Other investigators have shown that phenols can reduce compound I to compound II and then compound II to native catalase (34). By wave-scan spectroscopy, since more native catalase was identified at the end of the measurement interval in the presence of EG, our results demonstrate that EG can also perform this action. Compound I is a powerful oxidant, as exemplified by its ability to oxidize even H₂O₂ itself (11). Since EG would attenuate the formation of these active derivatives, this would provide another mechanism by which EG could prevent cardiovascular dysfunction in septic shock.

It is important to recognize, however, that while we showed that EG could inhibit H₂O₂ signaling in the in vitro preparation, this does not necessarily mean that this was the mechanism by which EG was operative in vivo or that this was the only mechanism involved. Phenolics are able to act as antioxidants in a number of ways (37). Phenolic hydroxyl groups are good hydrogen donors: hydrogen-donating antioxidants can react with reactive oxygen and reactive nitrogen species in a termination reaction. The antioxidant capacity of phenolic compounds is also attributed to their ability to chelate metal ions involved in the production of free radicals. Phenolic structures often have the potential to strongly interact with proteins because of their hydrophobic benzenoid rings and hydrogen-binding potential of the phenolic hydroxyl groups. We previously showed (27) that methylene blue could inhibit Lzm-S’s vasodilatory effect in the carotid artery preparation, and this may occur by methylene blue’s effect on decreasing cGMP, possibly through changes in the redox state of iron. It is possible that EG could work through a similar mechanism.

We recognize, moreover, that the nature of H₂O₂ signaling is often dependent on the preparation used. In our carotid artery preparation, we showed that when H₂O₂ concentrations >10⁻⁴ mol/l were administered a vasoconstrictor effect was observed, while at lower concentrations H₂O₂ caused vasodilation (27). On the other hand, in other preparations, there was primarily a vasoconstrictor effect that occurred (40). Further work must be done to completely understand EG’s mechanism of action in the in vivo preparation, since there may be mechanisms other than those considered that may have played a role in EG’s effects in the whole animal preparation.

It also appears that EG must encompass some unique geometric, chemical, and/or structural properties that allowed it to prevent Lzm-S’s in vitro effects, since similarly related phenols could not prevent them. EG is relatively nontoxic, and in the mouse the median lethal dose (LD₅₀) has been reported to be 6,000 mg/kg (material safety data sheet, Sigma). As part of this study, we additionally tried to determine the extent to which the type of ester attached to the gallate structure was a major determinant of EG in inhibiting Lzm-S-induced vasodilation in the in vitro preparation (see Preliminary Screened Compounds to Inhibit Lzm-S-Induced Vasodilation). We found that gallic acid produced no inhibitory effect on Lzm-S-induced vasodilation; that propyl gallate caused a possible inhibitory effect; and that methyl gallate produced a significant inhibitory effect. Thus the methyl ester of gallic acid as well as the ethyl ester can produce potent inhibiting effects in the in vitro preparation. We therefore think that both the type of ester and the gallate structure are important for inhibition of Lzm-S’s in vitro effects.

In the in vivo study, we gave a high concentration of EG based on the work of Gao et al. (13). Gao et al. (13) studied the metabolism of EG when fed intragastrically to rats. When EG was given at a concentration of 20 mg/kg to rats, they found that the peak plasma concentration was in the 10⁻⁴ mol/l range and that this concentration decreased to the 10⁻⁹ mol/l range at 1 h after administration. We thought that a slightly higher concentration of EG (i.e., between the 10⁻⁵ mol/l and 10⁻⁶ mol/l range) would be necessary to block Lzm-S’s cardiovascular effect in this in vivo preparation, so that we gave 40 mg·kg⁻¹·h⁻¹ in the present study. This would keep the serum EG concentrations in the desired range, since we recognized that EG must be rapidly metabolized or excreted. Furthermore, since the amount of EG contained in food substances is much less [for instance, in red wine the concentration is ~5 mg/l (32)], it appears unlikely that one could achieve the 10⁻⁶ mol/l serum concentration by ingesting EG-containing nutrients.

In this study, we used a bacteremic E. coli model to examine the effect of EG on cardiovascular effects in septic shock (26, 30), since this canine model was the original model that led to the discovery that Lzm-S was important in the development of cardiovascular collapse in this condition. We therefore thought that this model was the most appropriate to first test this hypothesis. Significantly, a primary objective of this study was to determine whether EG could reverse hemodynamics when the animal was overwhelmingly sick and when MAP was really low. Our results show that EG is capable of performing this feat in a whole animal preparation. Further work will be necessary to determine whether EG is beneficial in other models of septic shock.

We of course recognize that this canine E. coli model cannot be taken as one representative of the human condition. In human septic shock, hemodynamics in sepsis may deteriorate more gradually, and we do not claim that this is the case in the present model, although this bacteremic model could be comparable to what occurs in humans who have an infected implanted catheter, endocarditis, or severe infection with a high-grade bacteremia (1). It is noteworthy that the homology between human and canine Lzm-S is very high at ~80%, and both species of Lzm-S can intrinsically generate H₂O₂ leading to cardiovascular dysfunction in in vitro preparations (24, 27), while other lysozymes, such as that from chicken, have only 60% homology and cannot produce these effects. Future studies will be needed to correlate hemodynamics with Lzm-S concentrations in patients in septic shock.

In contrast to the beneficial effect of EG in our carotid artery preparation, we previously showed that other antioxidants, such as NAC, reduced glutathione (GSH), and sodium ascorbate (NaAc) could not prevent Lzm-S-induced vasodilation in this in vitro preparation (see Table 2 in Ref. 27). These agents cause a decrease in H₂O₂ by a manner different from that caused by EG. They serve as essential substrates for the enzymatic metabolism of H₂O₂ by peroxidases. Although these antioxidants would reduce the concentration of H₂O₂, they do not prevent the metabolism of H₂O₂ by catalase in the manner described for EG. The fact that antioxidants may differ in their ability to metabolize H₂O₂ in a slightly different manner was initially considered by Wolin and colleagues (4, 5). Although the mechanisms are not totally clear, these investigators found the metabolism of H₂O₂ by different types of enzymes to vary in the ability to stimulate formation of cGMP (see Table 2 in
Thus EG may have a beneficial effect compared with other Lzm-S’s ability to generate H$_2$O$_2$ in the carotid artery pre- 
paroxysm as a bioassay to establish that EG could inhibit Lzm-S’s 
effects of sepsis. Lzm-S is a product of lysis of leukocytes in 
sepsis model. In our model, despite the fact that EG restored 
survival as shown by Danner and colleagues (9) in a canine 
model or that EG inhibits all 
endotoxemia but more cardiovascular dysfunction and 
and DNA damage in MDCK cells. 
Characterization of membrane N-glycan binding sites of 
lysozyme for cardiac depression in sepsis. 
Histamine H$_3$ activation depresses cardiac function in experimental sepsis. 
Lysozyme, a mediator of sepsis that intrinsically generates hydrogen 
peroxide as a paracrine vascular 
mediator: regulation and signaling leading to dysfunction. 
Biesalski HK, McGregor GP. Antioxidant therapy in critical care—is the 
Burke TM, Wolin MS. Hydrogen peroxide elicits pulmonary arterial 
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6. Cai H. Hydrogen peroxide regulation of endothelial function: origins, 
7. Chance B. The primary and secondary compounds of catalase and methyl 
el the human condition must be 
considered as indicated by the elevated liver function tests, elevated 
other therapies would have to be administered to address these derangements 
in the clinical condition.

Septic shock still carries a very high mortality that 
approaches 40% in clinical medicine despite appropriate treat-
ment (1, 36). Newer treatments are desperately required. Al-
though antioxidants have been touted as a possible treatment in 
septic shock (3), in contrast to other investigators, we used 
Lzm-S’s ability to generate H$_2$O$_2$ in the carotid artery prepa-
ration as a bioassay to establish that EG could inhibit Lzm-S’s 
effects in vitro systems. We then showed that EG could reverse 
cardiovascular dysfunction in our in vivo septic model. 
Although H$_2$O$_2$ has been predominantly identified as a 
substrate that causes tissue injury due to peroxidation, recent 
information indicates that it is also a potent signaling mediator 
of inflammation as well (2, 6, 41). Although the relationship of 
animal models to the human condition must be considered 
cautiously, and although further work needs to be performed, 
these results suggest that compounds such as EG may be useful 
in the treatment of cardiovascular collapse in septic shock.

GRANTS

Supported by the Health Sciences Centre Foundation and the Biology of 
Breathing Group, Manitoba Institute of Child Health.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).


