Preconditioning with ethanol prevents postischemic leukocyte-endothelial cell adhesive interactions

TAJI YAMAGUCHI,1,2 CATHERINE DAYTON,1 T. SHIGEMATSU,1 PATSY CARTER,1 TOSHIKAZU YOSHIKAWA,2 DEAN C. GUTE,1 AND RONALD J. KORTHUIS1
1Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center, Shreveport, Louisiana 71130; and 2First Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan

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First published May 16, 2002; 10.1152/ajpheart.00173.2002.—Long-term ethanol consumption at low to moderate levels exerts cardioprotective effects in the setting of ischemia and reperfusion (I/R). The aims of this study were to determine whether I/R a single orally administered dose of ethanol [ethanol preconditioning (EtOH-PC)] would induce a biphasic temporal pattern of protection (early and late phases) against the inflammatory responses to I/R and 2) adenosine and nitric oxide (NO) act as initiators of the late phase of protection. Ethanol was administered as a bolus to C57BL/6 mice at a dose that achieved a peak plasma concentration of ~45 mg/dl 30 min after gavage and returned to control levels within 60 min of alcohol ingestion. The superior mesenteric artery was occluded for 45 min followed by 60 min of reperfusion beginning 10 min or 1, 2, 3, 4, or 24 h after ethanol ingestion, and the numbers of fluorescently labeled rolling and firmly adherent (stationary) leukocytes in single postcapillary venules of the small intestine were quantified using intravital microscopic approaches. I/R induced marked increases in leukocyte rolling and adhesion, effects that were attenuated by EtOH-PC 2–3 h before I/R (early phase), absent when assessed after 10 min, 1 h, and 4 h of ethanol ingestion, with an even more powerful late phase of protection reemerging when I/R was induced 24 h later. The anti-inflammatory effects of late EtOH-PC were abolished by treatment with adenosine deaminase, an adenosine A2 (but not A1) receptor antagonist, or a NO synthase (NOS) inhibitor during the period of EtOH-PC. Preconditioning with an adenosine A2 (but not an A1) receptor agonist in lieu of ethanol 24 h before I/R mimicked the protective actions of late phase EtOH-PC. Like EtOH-PC, the effect of preconditioning with an adenosine A2 receptor agonist was abrogated by coincident NOS inhibition. These findings suggest that EtOH-PC induces a biphasic temporal pattern of protection against the proinflammatory effects of I/R. In addition, our observations are consistent with the hypothesis that the late phase of EtOH-PC is triggered by NO formed secondary to adenosine A2 receptor-dependent activation of NOS during the period of ethanol exposure.

Epidemiological studies indicate that long-term, regular consumption of alcoholic beverages at low to moderate levels (1–3 drinks/day for months to years) decreases the incidence of coronary artery disease and improves survival in patients suffering myocardial infarctions (15, 36, 43, 49). While the mechanisms whereby regular alcohol ingestion affords protection against ischemia-reperfusion (I/R) injury are unclear, it has been suggested that the effects of ethanol on platelet function, plasma lipid levels, and fibrinolytic activity may play a role (16, 39, 42). More recent evidence indicates that chronic ethanol consumption is cardioprotective by a mechanism similar to that reported for acute or early phase ischemic preconditioning (IPC, a phenomenon wherein tissues are rendered resistant to the deleterious effects of prolonged I/R by antecedent exposure to short periods of vascular occlusion). That is, daily ethanol ingestion for 3–12 wk reduces infarct size in hearts subjected to I/R by a mechanism that is triggered by occupancy of adenosine A1 receptors, requires protein kinase C-ε as an obligatory downstream signaling element, and is mediated by activation of ATP-sensitive potassium channels (1, 7, 8, 12, 21, 22, 28, 30, 31, 34, 44, 53).

The aforementioned work indicates that, like IPC, long-term consumption of low to moderate levels of ethanol on daily basis induces an adaptive transformation to a defensive or protected phenotype that affords enhanced resistance to the deleterious effects of I/R. However, it is not clear whether such a preconditioned state would be invoked in naive (nondrinking) individuals by ingestion of ethanol as a single bolus [ethanol preconditioning (EtOH-PC)]. This is an important issue given the comparatively large segment of the population who rarely or only intermittently consumes alcoholic beverages. In addition, virtually no informa-
tion is available regarding the effect of antecedent ethanol exposure on I/R-induced leukocyte-endothelial cell adhesive interactions, inflammatory events that play a critical role in the reperfusion component of total tissue injury induced by I/R (1, 17, 18, 35). Thus the two major aims of the present study were to determine whether acute ethanol consumption (EtOH-PC) would prevent posts ischemic leukocyte-endothelial cell adhesive interactions and, if so, the time course over which EtOH-PC exerts its anti-inflammatory actions. Our results indicate that EtOH-PC produced a preconditioned state that exhibited a biphasic temporal pattern of expression. An early phase of partial protection (50–75% reduction in posts ischemic leukocyte-endothelial cell adhesive interactions) was invoked by ethanol ingestion 2–3 h before I/R. Although no antiadhesive effects were noted when I/R was induced 10 min, 1 h, or 4 h after alcohol consumption, the posts ischemic increases in leukocyte adhesion were completely prevented by EtOH-PC 24 h before I/R (late phase or delayed EtOH-PC).

Because the antiadhesive effects late EtOH-PC were so much more prominent than those manifested in the early phase, we focused on subsequent studies on identification of the initiators of this second window of protection. Our observations are consistent with the hypothesis that late EtOH-PC is triggered by nitric oxide (NO) formed secondary to adenosine A2 receptor-dependent activation of NO synthase (NOS) during the period of ethanol exposure.

METHODS

Animals

Male and female C57BL/6 mice (7–8 wk of age, weighing 18–22 g) were obtained from Jackson Laboratories (Bar Harbor, ME), maintained on standard mouse chow, and used at 7–10 wk of age (22–25 g). The experimental procedures described herein were performed according to the criteria outlined in the National Institutes of Health guidelines and were approved by the Louisiana State University Health Sciences Center-Shreveport Institutional Animal Care and Use Committee.

Ethanol Exposure

To determine the time course over which EtOH-PC was effective as a preconditioning stimulus, ethanol was instilled into the stomachs of conscious mice by gavage 10 min or 1, 2, 3, 4, or 24 h before the experiments. Pilot experiments were conducted to determine the volume of ethanol that would have to be administered by gavage to produce a peak increase in plasma ethanol to ~45 mg/dl, a plasma level equivalent to that achieved in humans consuming 1–2 alcoholic beverages. Plasma ethanol was determined using a colorimetric assay (Kit 332-C, Sigma; St. Louis, MO). From these experiments, we determined that the volume of 95% ethanol to be instilled (in μl) could be calculated as follows: [body weight (in g) × 0.6] + 0.3. This volume of ethanol was mixed in 0.3 ml of sterile distilled water just before administration to the mice as a bolus by gavage. Mice in the sham control (no I/R) and I/R alone (no EtOH-PC) groups received sterile distilled water without ethanol by gavage. All animals were then re-turned to their cages, where they had free access to food and water before the experiments.

Surgical Procedures and Induction of IIR

Sham and ethanol-treated mice were anesthetized initially with a mixture of ketamine (150 mg/kg body wt im) and xylazine (7.5 mg/kg body wt im). After a surgical plane of anesthesia was attained, a tracheotomy was performed to facilitate breathing during the experiment. The right carotid artery was cannulated, and systemic arterial pressure was measured with a Statham P23A pressure transducer (Gould) connected to the carotid artery catheter. Systemic blood pressure was recorded continuously with a personal computer (Power Macintosh 8600, Apple) equipped with an analog-to-digital converter (MP 100, Biopac Systems). The left jugular vein was also cannulated for administration of carboxyfluorescein diacetate, succinimydyl ester (CFDASE, Molecular Probes; Eugene, OR), a fluorescent dye that labels leukocytes. CFDASE was dissolved in DMSO at a concentration of 5 mg/ml and stored at −20°C until use. After these procedures, a midline abdominal incision was performed, and 10 min or 1, 2, 3, 4, or 24 h after ethanol was administered by gavage, the superior mesenteric artery was occluded with a microvascular clip for 0 (sham) or 45 min. After the ischemic period, the clip was gently removed, and leukocytes were labeled with CFDASE by intravenous administration of the fluorochrome solution (25 μg/ml saline) at 20 μl/min for 5 min. Leukocyte-endothelial cell adhesive interactions were observed over minutes 30–40 and 60–70 of reperfusion.

Intravital Fluorescence Microscopy

The mice were positioned on a 20 × 30-cm Plexiglas board in a manner that allowed a selected section of small intestine to be exteriorized and placed carefully and gently over a glass slide covering a 4 × 3-cm hole centered in the Plexiglas. The exposed small intestine was superfused with warmed (37°C) bicarbonate-buffered saline (BBS, pH 7.4) at 1.5 ml/min using a peristaltic pump (model M312, Gilson). The BBS was bubbled with a mixture of 5% CO2-95% N2 to reduce the oxygen tension to physiological intraperitoneal levels (40–50 mmHg). With the exception of the area to be observed by intravital microscopy, the exteriorized region of the small bowel was covered with BBS-soaked gauze and cellophane to minimize the tissue dehydration, temperature changes, and influence of respiratory movements. The superfusate was maintained at 37 ± 0.5°C by pumping the solution through a heat exchanger warmed by a constant-temperature circulator (model 1130, VWR). Body temperature of the mouse was maintained between 36.5 and 37.5°C with the use of a thermostatically controlled heat lamp. The board was mounted on the stage of an inverted microscope (DIAPHOT TMD-EF, Nikon), and the intestinal microcirculation was observed through a ×20 objective lens. Fluorescence images of the microcirculation (excitation wavelength, 420–490 nm; emission wavelength, 520 nm) were detected with a charge-coupled device (CCD) camera (XC-77, Hamamatsu Photonics), a CCD camera control unit (C2400, Hamamatsu Photonics), and an intensifier head (M4314, Hamamatsu Photonics) attached to the camera. Microfluorographs were projected on a television monitor (PVM-1953MD, Sony) and recorded on videotape using a videocassette recorder (HR-S4600U, JVC) for off-line quantification of measured variables during playback of the videotaped image. A video time-date generator (WJ810, Panasonic) displayed the stopwatch function onto the monitor.
The intravital microscopic measurements described below were obtained over minutes 30–40 and 60–70 of reperfusion or at equivalent time points in the control groups. The intestinal segment was scanned from the oral to aboral section, and 10 single, unbranched venules (20–50 μm diameter, 100 μm length) were observed, each for at least 30 s. Leukocyte-endothelial cell interactions (the numbers of rolling and firmly adherent leukocytes) were quantified in each of the 10 venules, followed by calculation of the mean value, which was used in the statistical analysis of the data. Circulating leukocytes were considered to be firmly adherent if they did not move or detach from the venular wall for a period ≥30 s. Rolling cells are defined as cells crossing an imaginary line in the microvessel at a velocity that is significantly lower than centerline velocity; their numbers were expressed as rolling cells per minute. The numbers of rolling or adhering leukocytes were normalized by expressing each as the number of cells per millimeter squared of vessel area.

Experimental Protocol

In initial studies, we characterized the time course for the protective actions of EtOH-PC by instilling ethanol into the stomach by gavage 10 min or 1, 2, 3, 4, or 24 h before I/R (n = 6 in each group). These studies revealed a biphasic pattern of protection with EtOH-PC. That is, I/R induced marked increases in leukocyte rolling and adhesion, effects that were attenuated by EtOH-PC 2–3 h before I/R (early phase) but absent when assessed 10 min or 1 or 4 h after ethanol ingestion. An even more powerful late phase of protection reemerged when I/R was induced 24 h after EtOH-PC. Because late-phase EtOH-PC completely prevented postschismic leukocyte rolling and adhesion, whereas early-phase EtOH-PC only attenuated these responses by 50% and 75%, respectively, we focused our remaining studies (groups 1–17, n = 6 in each group) on the factors responsible for triggering the late phase of EtOH-PC, which is apparent 24 h after ethanol ingestion. Figure 1 illustrates the general design of the experimental protocols for these latter studies, and a description for each group is presented below. Because male mice were used in all of the aforementioned groups, we also compared the effects of sham, I/R alone, and EtOH-PC + I/R in males and females (n = 6) to determine whether the sex of the animals influenced leukocyte-endothelial cell adhesive responses to these perturbations.

Group 1: sham. As a control for the effects of the gavage procedure on day 1 and for experimental duration on day 2, male or female mice in this group were administered sterile distilled water alone (without ethanol) by gavage on day 1 at a volume calculated as described above for mice receiving ethanol in distilled water. Twenty-four hours later (day 2), the small intestine was prepared for intravital microscopic observation of leukocyte-endothelial cell interactions, with all measurements obtained in continuously perfused venules at time points equivalent to those described for the I/R group (group 2).

Group 2: I/R alone. After administration of sterile distilled water by gavage on day 1, the small intestines of male or female mice in this group were subjected to 45 min of ischemia followed by 70 min of reperfusion (I/R) 24 h later (day 2), with all variables recorded during minutes 30–40 and 60–70 of reperfusion.

Group 3: EtOH-PC alone. The aim of the studies outlined for this group was to determine the effect of late EtOH-PC alone (no subsequent I/R) on leukocyte rolling and adhesion on day 2. Mice in this group received ethanol by gavage on day 1 but no I/R on day 2, with all variables recorded in continuously perfused venules on day 2 at time points equivalent to those described for the I/R group (group 2).

Group 4: EtOH-PC + I/R. To determine the effects of late EtOH-PC on I/R-induced leukocyte rolling and adhesion, male or female mice in this group received ethanol by gavage on day 1 and were subjected to I/R on day 2.

Group 5: acetaldehyde + I/R. A major reaction product of ethanol metabolism is acetaldehyde (25). Because acetaldehyde exerts a variety of effects in its own right (2, 3, 26, 46), we sought to determine whether the anti-inflammatory effects of ethanol exposure were due to this metabolite. Mice were treated with acetaldehyde (Sigma) by intraperitoneal injection on day 1 at a concentration (9.8 mM in 0.3 ml sterile saline) equal to the peak plasma ethanol concentration that was achieved in EtOH-PC animals. The small intestines of these animals were then subjected to I/R on day 2, with all variables measured at the time points outlined for group 2.

Identification of potential initiators of the beneficial actions of late-phase EtOH-PC required intraperitoneal administration of pharmacological antagonists and inhibitors according to a protocol that allowed these agents to target the respective putative triggers during the period of ethanol exposure. To accomplish this objective, the pharmacological agent of interest was injected into the peritoneal cavity 10 min before ethanol (or its vehicle) administration. The mice were then lightly anesthetized with ketamine (135 mg/kg body wt im) and xylazine (6.8 mg/kg body wt im) ~45 min after ethanol administration (55 min after treatment with the pharmacological agent of interest). Peritoneal cavity fluid was then removed by aspiration 60 min after ethanol instillation. The peritoneal cavity was then slowly flushed three times with 0.5 ml warmed (37°C) sterile saline via a syringe attached to a 16-gauge needle that was introduced through the abdominal wall. Before each flush was aspirated, the abdomen was gently massaged to assure adequate mixing, with care taken to avoid injury to the intestine by the needle. Upon completion of the lavage cycles, the needle was removed, and the incision was closed with 6-0 nylon sutures. After recovery from anesthesia, the animals were allowed to free access to water and standard chow. I/R was then induced on day 2. To control for any potential effects of the peritoneal lavage procedure, we repeated the studies outlined for groups 1, 2, and 4 in the mice undergoing peritoneal lavage (groups 6–8). The designation (L) after each of the descriptive names for groups 6–17 below refers to the fact that the peritoneal lavage procedure was used in each group.

Group 6: sham(L). As a time control for the potential effects of experimental duration and the peritoneal lavage procedure, mice in this group received 0.5 ml saline drug vehicle by intraperitoneal injection 10 min before the oral administration of sterile distilled water (ethanol vehicle) by gavage. Seventy minutes after intraperitoneal injection, three cycles of peritoneal lavage were performed, as described above. Twenty four hours later, the animals were prepared for intravital microscopic study, with all variables recorded in postcapillary venules at the times outlined for group 1 above.

Group 7: I/R(L) alone. Mice in this group were treated as described for group 2 except that the peritoneal lavage procedure was conducted as described above.

Group 8: EtOH-PC + I/R(L). The same protocol as described for group 4 was repeated in these studies except that the peritoneal lavage procedure was conducted as described above.

Group 9: adenosine deaminase + EtOH-PC + I/R(L). To test the hypothesis that adenosine serves as a trigger of the antiadhesive effects of late EtOH-PC, mice in this group
received adenosine deaminase (ADA; Sigma, 0.25 U/animal in 0.5 ml saline vehicle) by intraperitoneal injection 10 min before ethanol gavage. The peritoneal lavage procedure was performed 60 min after ethanol administration to wash out the ADA, as described above.

Group 10: L-N5-(1-iminoethyl)-ornithine + EthOH-PC + I/R(L). To determine whether NO served as an initiator of the beneficial effects of late EthOH-PC, mice in this group were treated with L-N5-(1-iminoethyl)-ornithine dihydrochloride (L-NIO; Calbiochem; San Diego, CA, 100 μM, 0.5 ml ip), a specific but non-isofrom-selective NOS inhibitor, 10 min before EthOH-PC, with subsequent removal of the agent by peritoneal lavage 60 min after gastric ethanol instillation, as described above. The small intestines of these animals were then subjected to I/R on day 2.

Group 11: EthOH-PC + ADA (1 h) + I/R and group 12: EthOH-PC + L-NIO (1 h) + I/R. To obtain additional support for the concept that development of the anti-inflammatory phenotype induced by late EthOH-PC was triggered by the adenosine and NO formed during the first hour after ethanol ingestion (the time frame over which plasma ethanol rises and falls after gastric instillation), we administered ADA or
l-NIO by intraperitoneal injection 1 h after gastric instillation of ethanol on day 1. The animals were then subjected to intestinal I/R on day 2. We postulated that such a protocol would fail to prevent the anti-inflammatory effects of EtOH-PC.

Groups 13 and 14: adenosine antagonist + EtOH-PC + I/R(L). The aim of these studies was to determine the adenosine receptor subtype that was responsible for initiating the beneficial actions of EtOH-PC. Ten minutes before ethanol gavage, mice in group 13 received 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; Research Biochemicals International (RBI); Natick, MA, 10 nM, 0.5 ml ip), a selective A1 adenosine receptor antagonist, whereas mice in group 14 received 3,7-dimethyl-1-propargylxanthine (DMPX; RBI, 10 nM, 0.5 ml ip), a selective A2 adenosine receptor antagonist. Sixty minutes after ethanol ingestion, these agents were washed out by peritoneal lavage on day 1, as described above. These animals were then subjected to intestinal I/R on day 2. Groups 15 and 16: adenosine antagonist + I/R(L). To obtain further support for the adenosine receptor subtype involved in initiating the protective actions of EtOH-PC, the small intestine of mice in groups 15 and 16 were pharmacologically preconditioned with selective adenosine A1 or A2 receptor agonists, in lieu of ethanol, on day 1. Mice in group 15 received N³-cyclopentyladenosine (CPA; RBI, 100 nM, 0.5 ml ip), a selective A1 adenosine receptor agonist, whereas mice in group 16 received N²-(2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl)adenosine (DMPA; RBI, 100 nM, 0.5 ml ip), a selective A2 adenosine receptor agonist, instead of ethanol. The adenosine receptor agonists were administered as described for the adenosine receptor antagonists in groups 12 and 13 above. After the agents were removed by peritoneal lavage on day 1, as described above, these animals were then subjected to I/R on day 2.

Groups 17: l-NIO + adenosine agonist + I/R(L). To address the hypothesis that ethanol-induced adenosine A2 receptor activation may stimulate NOS to produce NO, which subsequently serves as a downstream signaling element in the triggering mechanism for EtOH-PC, mice in this group were treated with l-NIO (100 μM, 0.5 ml ip) and DMPA (100 nM, 0.5 ml ip) 10 min after l-NIO, in lieu of ethanol. After the agents were removed by peritoneal lavage on day 1, as described above, these animals were then subjected to I/R on day 2.

The dosages of the drugs used herein were derived from previous reports [ADA (1, 11), l-NIO (27, 41), DPCPX (4, 11, 38), DMPX (4, 11, 38), CPA (45), and DMPA (51)].

Statistical Analysis

All values obtained in this study are expressed as means ± SE. The data were initially analyzed with standard statistical analyses, i.e., ANOVA. To identify which groups were statistically different, Scheffe’s post hoc test was employed. Statistical significance was defined at P < 0.05.

RESULTS

Mean arterial blood pressures were similar in all groups under baseline conditions before I/R, averaging ~100 mmHg. In the groups subjected to I/R, mean blood pressure decreased transiently by ~10 mmHg upon removal of the clamp on the superior mesenteric artery but returned to control levels within 5 min, where it remained for the remainder of the protocol (data not shown).

The time course for changes in plasma ethanol concentration after gastric instillation is depicted in Fig. 2. Ethanol was detectable in the plasma before gastric instillation, an effect that is likely due to ongoing production of the alcohol by enteric microorganisms followed by intestinal absorption (25). With the use of our dosing regimen, plasma ethanol increased to a peak value of ~45 mg/dl (~9.76 mM) 30 min after gavage and returned to control levels within 60 min of alcohol administration.

In initial studies, we characterized the temporal characteristics of the protection afforded by EtOH-PC by increasing the time interval between ethanol ingestion and the induction of I/R from 10 min to 1, 2, 3, 4 and 24 h. As shown in Fig. 3, A and B, I/R induced marked increases in the numbers of rolling and firmly adherent leukocytes. Although no protective effects were noted in animals that ingested ethanol 10 min, 1 h, or 4 h before I/R, postischemic leukocyte rolling and adhesion were reduced by ~50% and ~75%, respectively, when ethanol was consumed 2 or 3 h before I/R (early-phase EtOH-PC). An even more powerful late phase of protection was evident in animals subjected to I/R 24 h after ethanol ingestion. In the latter experiments, the postischemic increases in leukocyte rolling and adhesion on day 2 were completely prevented by EtOH-PC 24 h earlier (late-phase EtOH-PC). Female mice exhibited similar responses to male animals (Fig. 4) in the sham, I/R alone, and late EtOH-PC + I/R groups.

In view of the magnitude of protection afforded by late- vs. early-phase EtOH-PC, the remaining studies focused on identifying potential triggers of late EtOH-PC. To control for the effects of the experimental manipulations involved in administration of pharmacological agents (see METHODS), we repeated the studies outlined for groups 1, 2, and 4 (sham, I/R, and EtOH-PC + I/R) in animals also subjected to the intraperito-
neal vehicle injection/lavage procedure [sham(L), I/R(L), and EtOH-PC/L(I/R(L), groups 6–8, respectively] on day 1. Comparison of the data for these groups in Figs. 3 and 5, respectively, indicated that leukocyte rolling and adhesion on day 2 were not influenced by the drug vehicle injection/lavage procedure on day 1.

Figure 5 illustrates the changes in the number of rolling (A) and adherent (B) leukocytes 30 or 60 min after reperfusion on day 2 in animals treated with ADA (group 9) or L-NIO (group 10) during EtOH-PC or 1 h after gastric instillation of ethanol (i.e., after ethanol levels returned to control levels; Fig. 1). ADA and L-NIO, when present during the first hour after gastric ethanol instillation of ethanol on day 1 (EtOH-PC/L(I/R(L), EtOH-PC/L(I/R(L), respectively; Fig. 5), prevented the beneficial actions of EtOH-PC on postischemic leukocyte rolling and adhesion on day 2. However, the anti-inflammatory effects of EtOH-PC were not abrogated by treatment with these agents 1 h after ethanol consumption [EtOH-PC + ADA (1 h) + I/R(L), EtOH-PC + L-NIO (1 h) + I/R(L), respectively; Fig. 5]. Preconditioning with equimolar acetaldehyde (acetaldehyde + I/R), a metabolite generated from ethanol in vivo, 24 h before I/R failed to prevent the postischemic increases in leukocyte rolling and adhesion. These observations indicate that the expression of the anti-inflammatory phenotype on day 2 that is produced in response to EtOH-PC on day 1 is initiated or triggered by the formation of adenosine and NO during the first hour after ethanol ingestion, the time period over which the plasma ethanol concentration is elevated.

To gain additional support for the hypothesis that adenosine serves as a trigger of late EtOH-PC as well as to identify the adenosine receptor subtype involved in this process, the small intestine was exposed to specific receptor antagonists during the period of EtOH-PC (first hour after ethanol ingestion) on day 1 (groups 13 and 14). As a second approach, the small intestine was pharmacologically preconditioned with adenosine receptor agonists in lieu of ethanol on day 1 (groups 15 and 16). As shown in Fig. 6, blockade of adenosine A2 [EtOH-PC + A2 antagonist + I/R(L)] but not adenosine A1 [EtOH-PC + A1 antagonist + I/R(L)]...
Adenosine A2 receptor agonist for 1 h on day 1 prevented the leukocyte-endothelial cell adhesive interactions induced by I/R 24 h later. On the other hand, treatment with an adenosine A1 receptor agonist failed to mimic the protective effects of EtOH-PC in I/R. Interestingly, the anti-inflammatory effects of preconditioning with the A2 receptor agonist were abolished by coincident NOS inhibition [Fig. 6, A and B; L-NIO + A2 antagonist-PC + I/R(L), group 17], a result consistent with the hypothesis that the triggering mechanism for EtOH-PC involves adenosine A2 receptor-dependent stimulation of NOS, which produces NO as a downstream signaling element.

**DISCUSSION**

The results of a large number of studies indicate that reperfusion causes tissue injury that can be considerably greater than that caused by ischemia alone (17, 18, 22, 35). Recognition of the fact that activated neu-
trophils are largely responsible for the production of postischemic cellular dysfunction has led to a considerable research effort directed at evaluating the potential for inhibition of leukocyte-endothelial cell adhesive interactions as a novel approach to the treatment of reperfusion injury (1, 17, 18, 22, 35). One highly promising avenue for the development of such therapeutic interventions has sprung from the discovery that tissues can be preconditioned to resist the deleterious effects of prolonged I/R by prior exposure to brief periods of vascular occlusion (IPC) and other mildly noxious stimuli (e.g., endotoxin derivatives and heat shock) (1, 6, 8, 11, 12, 21, 22).

Epidemiological evidence suggests that chronic ingestion of relatively low quantities of ethanol induces a preconditioned state reminiscent of that produced by ischemic preconditioning (15, 36, 43, 49). That is, individuals who regularly consume 1–2 alcoholic beverages/day over the course of weeks to months demonstrate reductions in both the incidence and severity of myocardial infarction relative to those who abstain from drinking. With the use of animal models, several recent studies have reported infarct-sparing effects associated with regular ingestion of relatively low quantities of ethanol over a period of 3–12 wk (28, 30, 31, 34, 44, 53). However, the ability of ethanol to prevent the leukocyte-dependent reperfusion component of tissue injury induced by I/R was not evaluated as part of these studies. Nor was it clear from this work whether continued ethanol consumption over a prolonged time period (several weeks to months or years) was required to produce a preconditioned state or whether ethanol ingested as a single bolus in naive (nondrinkers) individuals would also confer protection against I/R [although there is evidence suggesting that intra-arterial infusion into intact hearts or direct application of ethanol onto cardiac myocytes in an acute setting produces a preconditioned state (7, 23, 24)]. Thus the first aim of our study was to determine whether ethanol ingestion would induce an anti-inflammatory preconditioned state when administered as a single bolus (EtOH-PC). We also sought to characterize the time course over which EtOH-PC exerted its protective effects. Our results indicate that EtOH-PC produced a preconditioned state that exhibited a biphasic temporal pattern of expression (Fig. 3). Although no protective effects were noted in animals treated with ethanol 10 min or 1 or 4 h before I/R, postischemic leukocyte rolling and adhesion were reduced by 50% and 75%, respectively, when EtOH-PC was initiated 2–3 h before I/R (early-phase EtOH-PC). An even more powerful late phase of protection was evident in animals subjected to I/R 24 h after ethanol ingestion. In the latter experiments, the postischemic increases in leukocyte rolling and adhesion on day 2 were completely prevented by EtOH-PC 24 h earlier. Because the numbers of rolling and adherent leukocytes were similar in male and female mice subjected to the sham procedure, I/R alone, or EtOH-PC + I/R, it does not appear that the responses to I/R alone or the effectiveness of late EtOH are influenced by the sex of the animals (Fig. 4).

The fact that the protective effects of EtOH-PC did not appear until plasma ethanol concentration returned to control levels were especially interesting in view of the work of Krenz et al. (23), who showed that ethanol induced the development of a cardioprotected state, but only if the alcohol was not present in the blood at the onset of ischemia. Moreover, the continued presence of ethanol during ischemia prevented the infarct-sparing effects of other preconditioning stimuli such as antecedent exposure to ischemia or mitochondrial ATP-sensitive potassium channel activation (23). On the basis of these results, it was suggested that the continued presence of ethanol may prevent preconditioning. Our results support this view because no protection against I/R was noted 10 min after ingestion. When examined 1 h after ethanol ingestion (a time point at which plasma alcohol levels had returned to control levels), EtOH-PC appeared to produce slight decreases in postischemic leukocyte rolling and adhesion, but these differences were not statistically significant. Because a protected phenotype did become apparent 2 h after ethanol administration, our results suggest that entrance into a preconditioned state by antecedent ethanol ingestion requires a longer time period for development than is required for IPC or intracoronary injection of ethanol, which arises within 10 min of the cycles of preconditioning ischemia or cessation of intra-arterial ethanol infusion, respectively.

Although a biphasic temporal pattern has also been noted for the expression of protected phenotypes after IPC in the heart (6, 8, 12, 21, 22), the early phase of IPC (acute or classical IPC) was more powerful in terms of the magnitude of its infarct-sparing effects relative to late (or delayed) IPC (6, 8, 12). The differences in the relative degree of protection afforded by early EtOH-PC vs. acute IPC and late EtOH-PC vs. delayed IPC suggest that these forms of preconditioning may be mechanistically distinct as well. Indeed, whereas both classical IPC and early-phase ethanol exposure induce preconditioned states in the myocardium, the latter does not involve oxidants or adenosine (23, 24). The mechanisms underlying late-phase EtOH-PC have not heretofore been examined.

Because the magnitude of the antiadhesive effects of antecedent ethanol exposure was so much greater when assessed 24 h after ingestion compared with earlier time points, we focused our subsequent studies on an examination of the mechanisms whereby late-phase EtOH-PC produced its postischemic anti-inflammatory effects. The fact that plasma ethanol concentrations peaked within 30 min and returned to control levels 60 min after ethanol ingestion indicates that the antiadhesive effects of EtOH-PC cannot be attributed to a direct effect of ethanol during I/R on day 2. Because ethanol is oxidatively metabolized by alcohol dehydrogenase to produce acetaldehyde (13, 14, 25), which demonstrates pharmacological properties in its own right (2, 3, 26, 46), we evaluated the effect of this metabolite as a preconditioning stimulus. However, acet-
aldehyde administration on day 1 (in lieu of ethanol) exerted no antiadhesive effects during I/R on day 2.

Our subsequent mechanistic studies involved intraperitoneal administration of a variety of pharmacological probes during discrete time points in the protocol, followed by removal of the agent of interest by peritoneal lavage. We conducted control experiments to exclude the possibility that the intraperitoneal injection/lavage procedure on day 1 did not exert a preconditioning effect after I/R on day 2. This was an important issue because the animals were lightly anesthetized during this procedure, and anesthetic agents have been shown to induce cardioprotective effects when administered as preconditioning stimuli (9, 20, 50). However, this preconditioning effect is limited to inhalational anesthetics such as isoflurane and sevoflurane and not injectable agents such as pentobarbital (9, 20). More recent work suggests that the protective actions of volatile anesthetics may relate to activation of ATP-sensitive potassium channels in mitochondria, an effect not shared with injectable agents (20). Our studies indicate that ketamine-xylazine injection by our protocol on day 1 does not induce the appearance of a late preconditioned state in the small intestine on day 2 [compare data presented in Figs. 3 (no injection/lavage) and 5 (with injection/lavage procedure)]. This anesthetic combination also fails to induce an early phase of preconditioning in rabbit hearts, in contrast to the infarct-sparing effects of volatile anesthetics (9). Our control experiments indicated that the injection/lavage procedure did not alter the responses to I/R alone or the effectiveness of late EtOH-PC.

It is well established that ethanol increases tissue adenosine levels, an effect that appears to be due to inhibition of the nucleoside transporter in plasma membranes (32). This observation led us to postulate that increased tissue levels of adenosine during the first hour after ethanol ingestion (the time frame over which plasma ethanol rises and falls) may serve to initiate or trigger the expression of the antiadhesive phenotype that becomes evident on subjecting the bowel to I/R 24 h later. To address this postulate, extracellular adenosine was metabolically inactivated during the period of ethanol exposure by intraperitoneal instillation of ADA (Fig. 5), an enzyme that catalyzes the deamination of the nucleoside to produce inosine. ADA treatment completely abolished the effects of EtOH-PC to prevent posts ischemic leukocyte rolling and adhesion on day 2. Because most of the ADA was removed by peritoneal lavage 1 h after ethanol ingestion, our findings support the notion that adenosine serves as a trigger for the development of late-phase EtOH-PC. More definitive evidence in favor of this concept is provided by the observation that administration of ADA 1 h after ethanol ingestion failed to prevent late EtOH-PC (Fig. 5).

To provide additional support for the concept that adenosine serves as an initiator of late EtOH-PC as well as to determine which adenosine receptor subtype was involved, we performed two additional series of experiments. First, the intestine was exposed to an adenosine A₁ or A₂ receptor antagonist during the period of EtOH-PC on day 1 (Fig. 6). In the second series, the ability of adenosine A₁ or A₂ receptor agonists to induce late preconditioning in lieu of ethanol was evaluated (Fig. 6). Our results indicate that the antiadhesive effects of late EtOH-PC were abolished by the presence of adenosine A₂ but not A₁ receptor antagonists (Fig. 6). On the other hand, preconditioning the small bowel with an adenosine A₂ receptor agonist (but not an adenosine A₁ agonist) on day 1 mimicked the protective effects of late EtOH-PC (Fig. 6). While these observations support the notion that adenosine triggers the development of an anti-inflammatory phenotype in response to ethanol by activating adenosine A₂ receptors, it remains unclear which A₂ receptor subtype (A₂&α vs. A₂β) predominates in this response.

Demonstrating a role for adenosine A₂ receptor activation as a key triggering event in late EtOH-PC was unexpected in view of the large number of studies supporting a role for adenosine A₁/A₃ receptors in initiating the infarct-sparing effects early and late IPC (5, 6, 8, 21, 22). Our findings were even more surprising in view of the fact that the cardioprotective effects induced by chronic ethanol consumption are dependent on adenosine A₁ receptors and are manifest 18 h after cessation of alcohol consumption (28, 30), which is nearly equivalent to the elapsed time between ethanol ingestion and induction of late-phase EtOH-PC (wherein ethanol is administered as a single bolus rather than by regular, long-term ingestion) in the present study. These discrepant findings may be related to induction of mechanistically distinct pathways for protection in response to different preconditioning stimuli. For example, acute exposure to ethanol in nondrinking individuals may induce a differential distribution between low- and high-affinity states of the various adenosine receptor subtypes relative to that occurring with chronic ethanol exposure (13, 14). It is also possible that the signaling pathway that induces the development of a protected phenotype in the vascular endothelium may differ from the cascade that triggers a preconditioned state in parenchymal cells (12). In support of the latter postulate, adenosine A₂ receptor activation triggers the appearance of a protected phenotype in cultured endothelial cells exposed to hypoxic preconditioning before prolonged hypoxia and reoxygenation (52). IPC also induces a preconditioned state in the hepatic sinusoidal endothelium that is dependent on ligation of adenosine A₂ receptors (4, 33). Finally, IPC-induced protection in splanchnic organs may be mechanistically distinct from the myocardium because stimulation of adenosine A₂ receptors appears to initiate the effects of early IPC in the liver (4, 33, 37, 38). Whatever the explanation, it is clear from our results that the antiadhesive effects of late EtOH-PC in the small intestine are initiated by activation of adenosine A₂ receptors during the period of ethanol exposure.

In addition to the demonstrated role for adenosine as trigger of late EtOH-PC, we also postulated that NO might serve as an initiator of this form of preconditioning.
ing because ethanol enhances both basal and flow-stimulated NOS activity and NO production in vivo and in cultured endothelial cells (10, 19). Moreover, there is evidence implicating this gaseous monoxide as a signaling molecule that triggers late IPC (6, 12, 21, 22). In support of this concept, we demonstrated that administration of the NOS antagonist L-NIO just before, but not 1 h after, ethanol administration on day 1 abolished the antiadhesive effects of late EtOH-PC on day 2 (Fig. 5). The latter observation supports the concept that NO plays an important role in initiating the antiadhesive actions in late EtOH-PC. We did not determine which NOS isoform was responsible for the production of the NO that triggers the development of late EtOH-PC. While the endothelial (eNOS), inducible (iNOS), and neuronal NOS (nNOS) isoforms can be expressed in the small intestine, iNOS is not constitutively present and requires several hours to reach full expression in response to appropriate stimuli (47). Thus the requirement for NO production during the first hour after ethanol ingestion to elicit a preconditioned state in postcapillary venules suggests that eNOS (or perhaps nNOS) is the most likely source of the NO that triggers late EtOH-PC. Studies involving isoform-selective inhibitors (which are not currently available for eNOS but are for iNOS) or eNOS knock-out animals may further clarify this issue.

While the factors responsible for increasing NOS activity in late EtOH-PC (or any form of preconditioning for that matter) are unknown, our observations that NOS inhibition was as effective as ADA or adenosine A2 receptor blockade in abolishing the protective effects of late EtOH-PC led us to hypothesize that adenosine and NO may serve as sequential triggering elements in the signaling cascade that induces the development of the protected phenotype rather than acting as independent initiators of this preconditioned state. Our finding that NOS inhibition prevented the late preconditioning induced by coadministration of an adenosine A2 receptor agonist is consistent with this postulate (Fig. 6). This notion is further supported by the observations that both ethanol and ligation of adenosine A2 receptors increase the activity of cAMP-dependent kinase, which in turn activates eNOS by phosphorylating Ser-1177 (13, 14, 29). The finding that adenosine stimulates L-arginine transport and NO biosynthesis by activation of A2 receptors on human umbilical vein endothelial cells (45) is also consistent with our conclusion that ethanol-induced adenosine A2 receptor activation may increase eNOS activity and NO production in intestinal postcapillary venules preconditioned with ethanol.

The signaling elements that are activated downstream from ethanol-induced NO formation and mediate the anti-inflammatory actions of late EtOH-PC are not clear. However, the signaling cascade underlying the cardioprotective effects of late IPC is better understood and may provide direction for future studies of the mechanisms whereby late EtOH-PC induces the development of a protected phenotype. According to the current paradigm (6, 12, 21, 22), late IPC is initiated by adenosine A1 receptor activation, oxidant formation, and the generation of NO. These triggers induce the sequential phosphorylation and activation of specific members of several kinase families (including protein kinase C, protein tyrosine kinases, and mitogen-activated protein kinases), signaling events that culminate in a nuclear factor-kB-dependent induction of cyclooxygenase-2 and iNOS gene expression. More recent work has implicated activation of the Janus kinase/signal transducer and activator of transcription pathway in the transcriptional upregulation of iNOS and protective effects of late IPC. As a consequence, the bioavailability of NO and prostanooids is restored or even enhanced in posts ischemic myocardium, where these biomolecules mediate the protective actions of late IPC. Upregulation of heat shock proteins, Bcl-2 (an antiapoptotic protein), and antioxidant enzymes may also play a role (6, 12, 21, 22). In addition to these transcription-dependent proteins, there is evidence suggesting that activation of ATP-sensitive potassium channels may mediate the infarct-sparing effects of late IPC (6, 12, 21, 22) and chronic ethanol consumption (34, 53). Whether late EtOH-PC induces the development of a protected phenotype by the same mechanisms or invokes novel signaling elements remains to be determined.

In summary, the results of this study indicate that antecedent ethanol exposure induces both an early and a late phase of preconditioning such that the small intestine is rendered resistant to the proinflammatory effects of I/R. The early phase becomes apparent 2 h after ethanol exposure, disappears 2 h later, and induces the development of a less effective protected phenotype than the late phase, which reemerges 24 h after ingestion of the alcohol. Although we did not examine the mechanisms underlying the development of the early phase of protection, our studies indicate that the late phase of EtOH-PC is triggered by formation of adenosine and NO during the period of ethanol exposure. Moreover, our observations are consistent with the notion that adenosine and NO may serve as sequential triggering elements in the signaling cascade that induces the development of the protected phenotype rather than acting as independent initiators of this preconditioned state. According to this scenario, tissue levels of adenosine increase during the period of ethanol exposure. Subsequent ligation of adenosine A2 receptors leads to activation of NOS (most likely eNOS) and the formation of NO, which acts as a downstream signaling element to initiate the anti-inflammatory effects of late-phase EtOH-PC, which become apparent 24 h after ethanol ingestion.

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