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# Adenosine Type 1 (A$_1$) Receptors Mediate Protection Against Myocardial Infarction Produced by Chronic, Intermittent Ingestion of Ethanol in Dogs

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## Abstract

*Background*: Chronic consumption of small amounts of ethanol protects myocardium from ischemic injury. We tested the hypothesis that adenosine type 1 ($A_1$) receptors mediate these beneficial effects. *Methods*: Dogs (*n*=37) were fed with ethanol (1.5 g/kg) or water mixed with dry food twice per day for 12 weeks, fasted overnight before experimentation, and instrumented for measurement of hemodynamics. Dogs received intravenous drug vehicle (50% polyethylene glycol in 0.1 N sodium hydroxide and 0.9% saline over 15 min) or the selective $A_1$ receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.8 mg/kg over 15 min) and were subjected to a 60 min coronary artery occlusion followed by 3 h of reperfusion. Myocardial infarct size and transmural coronary collateral blood flow were measured with triphenyltetrazolium chloride staining and radioactive microspheres, respectively. *Results*: The area at risk (AAR) for infarction was similar between groups. Pretreatment with ethanol significantly reduced infarct size to 13±2% (*n*=7) of the AAR as compared to control experiments (26±2%; *n*=7). DPCPX abolished the protective effects of ethanol pretreatment (30±3%; *n*=7) but had no effect in dogs that did not receive ethanol (25±2%; *n*=7). No differences in transmural coronary collateral blood flow were observed between groups. *Conclusions*: The present findings indicate that chronic ingestion of small amounts of ethanol produces myocardial protection that persists after the discontinuation of ethanol. The results indicate that $A_1$ receptors mediate ethanol-induced preconditioning in dogs independent of alterations in systemic hemodynamics or coronary collateral blood flow.

## Keywords

## 1. Introduction

Chronic consumption of small amounts of ethanol improves survival in patients after acute myocardial infarction [1], [2], but the mechanisms responsible for this beneficial effect are incompletely described. We [3], [4] and others [5] have recently demonstrated that chronic, moderate ethanol consumption preserves myocardium from irreversible ischemic damage by activation of mitochondrial adenosine triphosphate-dependent potassium ($K_{ATP}$) channels, a process that we termed 'chronic ethanol-induced preconditioning' (CEPC). Mitochondrial $K_{ATP}$ channels [6] also mediate the protective effects of ischemic preconditioning (IPC) through an intracellular signal transduction pathway that includes adenosine type 1 ($A_1$) receptors [7] coupled to inhibitory guanine ($G_i$) nucleotide binding proteins [8] and protein kinase C (PKC) [9].

Evidence initially obtained in isolated guinea pig and rat hearts indicates that chronic exposure to small amounts of ethanol protects myocardium from subsequent ischemic injury by a signal transduction pathway similar to that implicated in IPC [10], [11], [12]. The salutary action of chronic ingestion of low doses of ethanol was attributed to activation of adenosine or alpha-adrenergic receptors [10], [12] and sustained translocation of the epsilon isoform of PKC [11]. However, hemodynamics and regional myocardial perfusion were not quantified nor were plasma ethanol concentrations assessed in these previous studies [10], [11], [12]. In contrast, we [3], [4] have employed a unique canine model of intermittent ethanol ingestion during the pretreatment period [13] that more closely resembles moderate ethanol consumption with meals in humans and consistently produces subintoxicating plasma ethanol concentrations [3]. Acute experimentation after ethanol pretreatment in this large animal model allows invasively-derived assessment of systemic hemodynamics and precise quantification of coronary collateral blood flow before and during prolonged coronary artery occlusion and reperfusion in vivo. In the present investigation, we tested the hypothesis that $A_1$ receptors mediate the protective effects of chronic intermittent ingestion of ethanol independent of alterations in hemodynamics or transmural myocardial perfusion.

## 2. Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin. All conformed to the Guiding Principles in the Care and Use of Animals of the American Physiological Society and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Revised, 1996).

### 2.1. General preparation

Mongrel dogs weighing between 25 and 30 kg were randomly assigned to receive dry dog chow (Lab Canine Diet, Richmond, IN) mixed with ethanol (1.5 g/kg) or an equal volume of water twice a day for 12 weeks [3], [4], [13]. Drinking water was provided ad libitum. We [3] have previously shown that administration of ethanol using this method produces peak blood ethanol concentrations of 52±4 mg/dl 45 min after eating. Dogs that did not consume the chow–ethanol mix or lost weight during the 12 week pretreatment period were excluded from further experimentation. Dogs were fasted overnight before and did not receive ethanol on the day of experimentation.

### 2.2. Implantation of instruments

Dogs were anesthetized with sodium barbital (200 mg/kg) and sodium pentobarbital (15 mg/kg) and ventilated with an air and oxygen mixture (fraction of inspired oxygen=0.25) after intubation of the trachea as previously described [3], [14]. Acid–base status and arterial blood gas tensions were maintained within the normal range by adjustment of respiratory rate and tidal volume throughout the experiment. After calibration, a double pressure transducer-tipped catheter was inserted into the aorta and left ventricle (LV) through the left carotid artery to measure arterial and LV pressures. The maximal rate of increase of LV pressure ($+dP/dt_{max}$) was obtained by electronic differentiation of the LV pressure wave form. The femoral artery and vein were cannulated for the withdrawal of reference blood flow samples and fluid administration, respectively. A thoracotomy was performed in the left fifth intercostal space. A heparin-filled catheter was inserted into the left atrial appendage for administration of radioactive microspheres. A 1.0 cm segment of the left anterior descending coronary artery (LAD) was dissected immediately distal to the first diagonal branch, and a silk ligature was placed around this vessel for production of coronary artery occlusion and reperfusion. The appearance of cyanosis and regional dyskinesia of myocardium immediately distal to the ligature were used to verify the adequacy of LAD occlusion in all experiments. Hemodynamic data were continuously monitored throughout the experiment, recorded on a polygraph, and digitized using a computer interfaced with an analog-to-digital converter.

## 2.3. Measurement of myocardial infarct size

At the conclusion of each experiment, the LAD was again occluded and cannulated at the occlusion site [14]. Briefly, 10 ml each of saline and patent blue dye were injected at equal pressure in the LAD and left atrium to delineate the anatomic area at risk (AAR) and the normal zone, respectively. The heart was fibrillated, removed, and sliced into serial 6 to 7 mm wide transverse sections. The unstained AAR was separated from the normal area, and the two regions were incubated for 20 min at 37 °C in 1% 2,3,5-triphenyltetrazolium chloride in 0.1 M phosphate buffer adjusted to pH=7.4. Infarcted and noninfarcted myocardium within the AAR were separated and weighed after being stored overnight in 10% formaldehyde. Infarct size was expressed as a percentage of AAR.

## 2.4. Measurement of regional myocardial perfusion

Carbonized plastic microspheres [15±2 μm (SD)] labeled with $^{95}$Nb, $^{141}$Ce, and $^{103}$Ru were used to measure myocardial perfusion as previously described [14]. Briefly, microspheres were administered into the left atrium as a bolus and flushed in with 10 ml of warm (37 °C) saline. A few seconds before injection, a timed collection of reference arterial blood flow was started from the femoral arterial catheter at a rate of 7 ml/min for 3 min. Microspheres were injected 5 min before LAD occlusion (after administration of vehicle or DPCPX), 30 min after LAD occlusion, and 1 h after final reperfusion. Transmural tissue samples were selected from the ischemic region and subdivided into subepicardial, midmyocardial, and subendocardial layers of approximately equal thickness. Samples were weighed and placed in scintillation vials, and the activity of each isotope was determined. Similarly, the activity of each isotope in the reference blood flow sample was assessed. Tissue blood flow (ml/min per g) was calculated as $Q_r \cdot C_m / C_r$, where $Q_r$ is the rate of withdrawal of the reference blood flow sample (ml/min), $C_m$ is the activity (cpm/g) of the myocardial tissue sample, and $C_r$ is the activity of the reference blood flow sample. Transmural blood flow was considered to be the average of the subepicardial, midmyocardial, and subendocardial blood flows.

## 2.5. Experimental protocol

Baseline hemodynamics were recorded 90 min after completion of the surgical preparation. All dogs were subjected to a 60 min LAD occlusion followed by 3 h of reperfusion. In four separate groups of experiments, dogs that consumed ethanol or water (control) mixed with dog chow for 12 weeks were randomly assigned to receive intravenous infusions of drug vehicle (50% polyethylene glycol in 0.1 N sodium hydroxide and 0.9% saline over 15 min) or the selective $A_1$ receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.8 mg/kg over 15 min) 60 min before LAD occlusion and reperfusion. We have previously shown that this dose of DPCPX markedly attenuates enhanced functional recovery of stunned myocardium produced by the volatile anesthetic isoflurane in dogs [15]. Dogs that developed intractable ventricular fibrillation and those with a subendocardial coronary collateral blood flow=0.15 ml/min per g were excluded from subsequent data analysis [16].

## 2.6. Statistical analysis

Statistical analysis of data within and between groups was performed with analysis of variance (ANOVA) with repeated measures followed by Student's $t$-test with Bonferroni's correction for multiplicity. Linear regression analysis was performed to determine the relationship between transmural collateral blood flow and myocardial infarct size. Changes within and between groups were considered statistically significant when $P<0.05$. Data are expressed as mean±standard error of the mean (S.E.M.).

# 3. Results

All dogs consumed the assigned diet during the 12 week pretreatment period. Thirty seven dogs were used to obtain 28 successful experiments. Four dogs were excluded from analysis because of intractable ventricular fibrillation during LAD occlusion or reperfusion (1 control, 2 ethanol pretreatment alone, 1 ethanol pretreatment

and DPCPX). Five dogs were excluded because subendocardial coronary collateral blood flow exceeded 0.15 ml/min/g (1 control, 1 DPCPX alone, 1 ethanol pretreatment alone, 2 ethanol pretreatment and DPCPX).

No differences in baseline systemic hemodynamics were observed between experimental groups (Table 1).

Table 1. Systemic hemodynamics

| | Baseline | Vehicle or OPCPX | Coronary occlusion | Reperfusion (min) 60 | 120 | 180 |
|---|---|---|---|---|---|---|
| HR (min$^{-1}$) | | | | | | |
| Vehicle | 128±4 | 128±4 | 117±7 | 117±6 | 120±4 | 122±5 |
| DPCPX | 129±7 | 126±7 | 127±5 | 126±3 | 126±5 | 121±6 |
| Ethanol+vehicle | 127±4 | 127±4 | 121±4 | 117±4 | 118±4 | 119±6 |
| Ethanol+DPCPX | 134±4 | 132±5 | 133±5 | 123±4 | 126±4 | 127±4 |
| | | | | | | |
| MAP (mmHg) | | | | | | |
| Vehicle | 104±3 | 104±3 | 88±3* | 95±7 | 98±5 | 97±4 |
| DPCPX | 109±3 | 110±3 | 89±3* | 90±2* | 89±3* | 91±2* |
| Ethanol+vehicle | 111±6 | 111±6 | 97±7 | 96±6* | 100±5 | 101±5 |
| Ethanol+DPCPX | 107±3 | 112±4 | 90±5 | 85±5* | 92±6 | 94±4 |
| | | | | | | |
| LVSP (mmHg) | | | | | | |
| Vehicle | 117±3 | 117±3 | 93±4* | 100±7 | 104±6 | 102±5 |
| DPCPX | 120±3 | 122±4 | 96±3* | 93±3* | 90±4* | 96±2* |
| Ethanol+vehicle | 122±7 | 122±7 | 105±6* | 99±5* | 104±4* | 106±4* |
| Ethanol+DPCPX | 118±4 | 125±4 | 84±6 | 87±6* | 95±7* | 98±5 |
| | | | | | | |
| LVEDP (mmHg) | | | | | | |
| Vehicle | 6±1 | 6±1 | 11±2* | 15±2* | 14±2* | 15±1* |
| DPCPX | 5±1 | 6±1 | 15±3* | 13±2* | 11±3 | 12±3 |
| Ethanol+vehicle | 7±1 | 7±1 | 11±2* | 8±2 | 9±2 | 8±2 |
| Ethanol+DPCPX | 5±1 | 6±1 | 15±2* | 17±2* | 18±2* | 14±2* |
| | | | | | | |
| +d$P$/d$t_{max}$ (mmHg·s$^{-1}$) | | | | | | |
| Vehicle | 1892±159 | 1892±159 | 1561±53 | 1445±70 | 1504±108 | 1279±55* |
| DPCPX | 1967±120 | 1927±114 | 1530±72* | 1576±51* | 1555±59* | 1456±70* |
| Ethanol+vehicle | 1921±172 | 1921±172 | 1484±172* | 1366±83* | 1337±102* | 1435±84* |
| Ethanol+DPCPX | 1922±111 | 1915±118 | 1663±191 | 1362±107* | 1470±103* | 1480±99* |

Data are mean±SEM; $n$=7 in each group.
*Significantly ($P$<0.05) different from vehicle or DPCPX.
Abbreviations: HR=heart rate; MAP=mean artorial pressure; LVSP and LVEDP=left ventricular systolic and end-diastolic pressures, respectively; +d$P$/d$t_{max}$=peak rate of increase of left ventricular pressure; DPCPX=8-cyclopentyl-1,3-dipropylxanthine.


Twice daily consumption of ethanol for 12 weeks did not affect baseline systemic hemodynamics. Intravenous administration of drug vehicle or DPCPX in the presence or absence of ethanol pretreatment was also devoid of hemodynamic effects. Significant ($P$<0.05) increases in LV end-diastolic pressure and decreases in mean arterial and LV systolic pressures and LV+d$P$/d$t_{max}$ were observed during LAD occlusion and reperfusion. Heart rate was unchanged during LAD occlusion and reperfusion. No differences in systemic hemodynamics were observed between groups during occlusion or reperfusion.

The AAR was similar between groups (vehicle 40±1; DPCPX 42±3; ethanol pretreatment and vehicle 40±2; ethanol pretreatment and DPCPX 42±2% of the LV). Twelve week pretreatment with ethanol significantly reduced myocardial infarct size to 13±2% of the AAR (Fig. 1, Fig. 2) as compared to control experiments (26±2%).
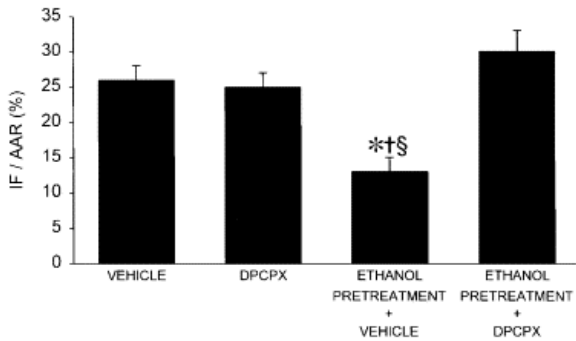


Fig. 1. Myocardial infarct size (IF) expressed as a percentage of the area of the left ventricle at risk (AAR). Abbreviation: DPCPX=8-cyclopentyl-1,3-dipropylxanthine. *Significantly ($P<0.05$) different from VEHICLE; †Significantly ($P<0.05$) different from DPCPX; §Significantly ($P<0.05$) different from ETHANOL+DPCPX.
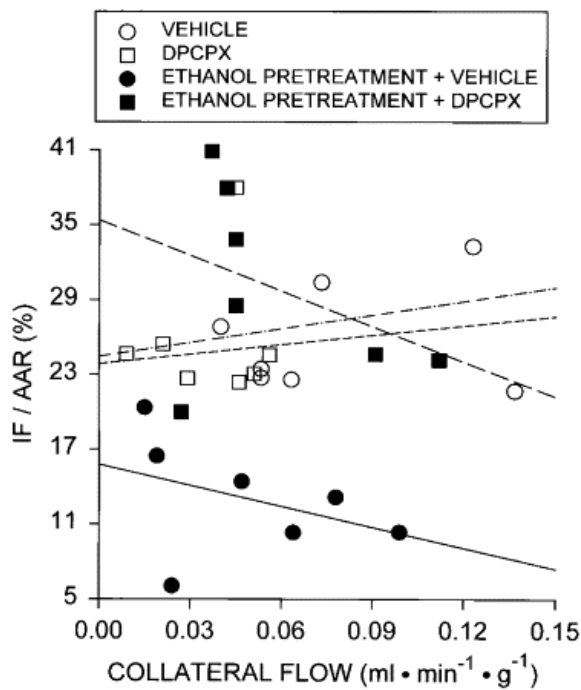


Fig. 2. Relationship between myocardial infarct size (IF) expressed as a percentage of the left ventricular area at risk (AAR) and transmural coronary collateral blood flow in dogs receiving VEHICLE (open circles), DPCPX (8-cyclopentyl-1,3-dipropylxanthine; open squares), ETHANOL+VEHICLE (solid circles), or ETHANOL+DPCPX (solid squares). Regression relationships between IF/AAR and collateral flow are illustrated for VEHICLE (line with short dashes; $y=25.6x+23.9$, $r=0.21$; $P=$NS), DPCPX (dash-dot line; $y=37.1x+24.5$, $r=0.12$; $P=$NS), ETHANOL+VEHICLE (solid line; $y=-55.8x+15.8$, $r=-0.39$; $P<0.05$), and ETHANOL+DPCPX (line with long dashes; $y=-94.5x+35.4$, $r=-0.38$; $P<0.05$). Note that the ETHANOL+VEHICLE regression line is shifted downward ($P<0.05$) compared with the regression lines for VEHICLE, DPCPX, and ETHANOL+DPCPX.

DPCPX abolished the protective effects of ethanol pretreatment (30±3%) but had no effect in dogs that did not receive ethanol (25±2%). Chronic intermittent ethanol ingestion for 12 weeks did not affect baseline regional myocardial perfusion. No differences in transmural coronary collateral blood flow (Table 2) were observed between groups.

Table 2. Transmural myocardial blood flow in the ischemic and normal regions (ml/min/g)

| | Vehicle of DPCPX | Coronary occlusion | Reperfusion |
|---|---|---|---|
| Ischemic region | | | |
| Vehicle | 1.08±0.11 | 0.08±0.01* | 1.22±0.14 |
| DPCPX | 1.25±0.20 | 0.04±0.01* | 1.58±0.31 |
| Ethanol+vehicle | 0.88±0.15 | 0.05±0.01* | 0.90±0.11 |
| Ethanol+DPCPX | 0.80±0.05 | 0.06±0.01* | 1.00±0.15 |
| | | | |
| Normal region | | | |
| Vehicle | 1.22±0.11 | 1.12±0.11 | 1.23±0.14 |
| DPCPX | 1.55±0.27 | 1.21±0.24 | 1.47±0.30 |
| Ethanol+vehicle | 1.34±0.17 | 0.98±0.14 | 0.94±0.11 |
| Ethanol+DPCPX | 1.05±0.16 | 0.93±0.11 | 1.18±0.14 |

Data are mean±SEM; $n$=7 in each group.
*Significantly ($P<0.05$) different from vehicle or DPCPX.
Abbreviation: DPCPX=8-cyclopentyl-1,3-dipropylxanthine.

# 4. Discussion

Previous investigations from this [3], [4] and other laboratories [5], [10], [11], [12], [17] have demonstrated that chronic, moderate ethanol consumption protects myocardium from ischemic injury in isolated and intact hearts. The results of these experiments obtained in several animal species are supported by recent clinical observations that chronic ingestion of small amounts of ethanol enhances survival after myocardial infarction in humans [1], [2]. The precise mechanisms responsible for this beneficial effect remain to be completely defined, but a signal transduction cascade has been implicated in CEPC that is remarkably similar to a major pathway responsible for IPC. The present results confirm the previous findings indicating that chronic, intermittent ethanol ingestion reduces the extent of myocardial infarction associated with prolonged coronary artery occlusion and reperfusion. The reduction in infarct size produced by chronic ethanol ingestion observed in dogs in the present and previous studies [3], [4] occurred independent of alterations in systemic hemodynamics and coronary collateral blood flow. Other studies conducted in rats [5], [12], [17] and guinea pigs [10], [11], [12] did not quantify transmural myocardial perfusion during experimentation or measure blood ethanol concentrations during pretreatment. Although we did not specifically determine blood ethanol concentrations during ethanol pretreatment in the present investigation, we have previously demonstrated that administration of ethanol using the methods described produces subintoxicating peak blood ethanol concentrations [3], [4].

The present results also demonstrate that the beneficial effect of ethanol was blocked by administration of the selective $A_1$ antagonist DPCPX before LAD occlusion, suggesting that CEPC is mediated by $A_1$ receptors in dogs. These findings support and extend the results of Miyamae et al. [10], [12] that also demonstrated a role for adenosine receptors in chronic ethanol-induced protection against myocardial ischemic injury. Isolated hearts from guinea pigs exposed to low concentrations (2.5 or 5%) of ethanol in their drinking water for 12 weeks before experimentation demonstrated improved functional recovery (peak developed LV pressure) and decreased myocardial necrosis (creatine kinase release) after 45 min of no-flow ischemia as compared with controls [10], [12]. These salutary effects were inhibited by the nonselective adenosine receptor antagonist 8-($p$-sulfophenyl)theophylline (SPT) and DPCPX but not by the selective $A_2$ antagonist 3,7-dimethyl-1-propargylxanthine (DMPX) [10], [12]. The results with a 12 week exposure to ethanol were similar to those obtained with IPC produced by 2 min of global ischemia followed by 5 min of reperfusion before no-flow

ischemia in the absence of ethanol pretreatment. The present and previous [10], [12] results are also partially supported by recent findings demonstrating that adenosine-induced myocardial protection against excessive catecholamine stimulation may be enhanced by chronic ethanol consumption in rats [18]. Hearts chronically exposed to ethanol demonstrated increased sensitivity to the protective effects of adenosine despite reductions in total myocardial adenosine release measured in coronary venous blood. These findings suggested that chronic ethanol ingestion may produce an upregulation or enhanced responsiveness of myocardial adenosine receptors [18]. Such phenomena may be associated with increased myocardial protection against ischemic injury because of the well-established link between $A_1$ receptors and $K_{ATP}$ channels [7], [19]. This intriguing hypothesis will require additional research to confirm, however. Extrapolation of the conclusions of Fenton and Chung [18] to the present results should also be qualified because larger amounts of ethanol (36% of total daily caloric intake) were chronically ingested for a more prolonged duration (8 months) by rats in this previous study. Whether administration of DPCPX during the preconditioning period prevents the development of CEPC also remains unknown.

In contrast to the continuous administration of ethanol in drinking water described in many previous studies [5], [10], [11], [12], [17], the present investigation used intermittent administration of ethanol with ad libitum access to fresh drinking water during the pretreatment period. Myocardial protection associated with this method of administration occurred after ethanol had been withheld the night before experimentation, in contrast to the presence of an average blood ethanol concentration of 3 mM immediately before experimentation previously described in one study [5]. Acute exposure to ethanol concentrations as low as 10 mM has been shown to produce direct protective effects against ischemic damage in ventricular myocytes and isolated hearts [20], [21] and enhance the functional recovery of stunned myocardium in vivo [22]. There is also experimental evidence in neural tissue indicating that acute administration of ethanol may directly activate $A_1$ receptors [23] by increasing the extracellular concentration of adenosine [24]. Thus, it remains possible that previous results implicating the $A_1$ receptor in CEPC obtained in small mammals continuously exposed to ethanol may have been influenced to some degree by the presence of ethanol immediately before experimentation. However, acute administration of intoxicating doses of ethanol immediately before coronary artery occlusion did not reduce infarct size in similar canine model [25]. These findings suggest that chronic ethanol ingestion may be required for myocardial protection to occur.

The present results should be interpreted within the constraints of several potential limitations. The specificity of DPCPX for the $A_1$ receptor has not been definitively established in canine myocardium, but this compound has been shown to be 700 times more selective for $A_1$ versus $A_2$ receptors using radioligand binding studies and in vitro functional assays [26]. Previous studies also indicate that the dose of DPCPX (0.8 mg/kg) used in the present investigation attenuates isoflurane-induced improvements in the functional recovery of stunned myocardium [15] and abolishes IPC in the canine heart [19] via $A_1$ receptor blockade. It is unlikely that myocardial protection produced by chronic, intermittent ethanol ingestion and the abolition of this beneficial effect by DPCPX were related to differential alterations in myocardial oxygen consumption because systemic hemodynamics were very similar between experimental groups. Nevertheless, coronary sinus oxygen tension was not specifically measured and myocardial oxygen consumption was not directly calculated in the present investigation. Whether DPCPX acutely alters the well-known beneficial effects of chronic, intermittent ethanol ingestion on platelet function and lipid metabolism also cannot be completely excluded as a potential explanation for observed findings. The present results also require qualification because oral ingestion of the dose of ethanol used in this investigation may produce greater blood ethanol concentrations in humans than dogs, although the pharmacokinetics of ethanol appear to be relatively similar between these species.

In summary, the present results demonstrate that chronic, intermittent consumption of small amounts of ethanol reduce myocardial infarct size in dogs independent of alterations in hemodynamics or transmural

myocardial perfusion. This protective effect was abolished by the selective $A_1$ antagonist DPCPX, indicating that the $A_1$ receptor mediates CEPC in vivo.

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