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## Moderate Ethanol Ingestion and Cardiovascular Protection:

### From Epidemiologic Associations to Cellular Mechanisms

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### Abstract

While ethanol intake at high levels (3-4 or more drinks), either in acute (occasional binge drinking) or chronic (daily) settings, increases the risk for myocardial infarction and ischemic stroke, an inverse relationship between regular consumption of alcoholic beverages at light to moderate levels (1-2 drinks per day) and cardiovascular risk has been consistently noted in a large number of epidemiologic studies. Although initially attributed to polyphenolic antioxidants in red wine, subsequent work has established that the ethanol component contributes to the beneficial effects associated with moderate intake of alcoholic beverages regardless of type (red versus white wine, beer, spirits). Concerns have been raised with regard to interpretation of epidemiologic evidence for this association including heterogeneity of the reference groups examined in many studies, different lifestyles of moderate drinkers versus abstainers, and favorable risk profiles in moderate drinkers. However, better controlled epidemiologic studies and especially work conducted in animal models and cell culture systems have substantiated this association and clearly established a cause and effect relationship between alcohol consumption and reductions in tissue injury induced by ischemia/reperfusion (I/R), respectively. The aims of this review are to summarize the epidemiologic evidence supporting the effectiveness of ethanol ingestion in reducing the likelihood of adverse cardiovascular events such as myocardial infarction and ischemic stroke, even in patients with co-existing risk factors, to discuss the ideal quantities, drinking patterns, and types of alcoholic beverages that confer protective effects in the cardiovascular system, and to review the findings of recent experimental studies directed at uncovering the mechanisms that underlie the cardiovascular protective effects of antecedent ethanol ingestion. Mechanistic interrogation of the signaling pathways invoked by antecedent ethanol ingestion may point the way towards development of new therapeutic approaches that mimic the powerful protective effects of socially relevant alcohol intake to limit I/R injury, but minimize the negative psychosocial impact and pathologic outcomes that also accompany consumption of ethanol.

### Keywords

ischemia/reperfusion; ethanol preconditioning; alcohol; myocardial infarction; stroke, coronary disease; inflammation; signaling; epidemiology

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## 1. Introduction

A large number of epidemiologic studies suggest that regular ingestion of alcoholic beverages at low to moderate levels (1-2 drinks per day) may induce tolerance to ischemia, even in the presence of co-existing risk factors. Subsequent studies conducted in a variety of animal models support this contention and have provided important mechanistic insight that may allow for development of new therapeutic strategies to limit I/R injury that remain effective despite the presence of co-morbid risk factors in humans. The development of such alternate pharmacologic approaches is an important focus of scientific inquiry because compliance with other cardioprotective lifestyle interventions such as exercise is often difficult to achieve, while use of alcoholic beverages is contraindicated for individuals susceptible to the development of alcoholism, especially the large majority who cannot be reliably identified by objective criteria (such as familial alcoholism) as being at risk prior to beginning ethanol consumption. In addition, ethanol ingestion increases the morbidity and mortality associated with hemorrhagic stroke, induces liver injury, aggravates high-fat diet-induced steatohepatitis, is toxic to neurons, increases the risk for development of breast, oral and gastrointestinal cancers, and has psychosocial consequences (e.g., impaired driving ability and performance of employment responsibilities), even when consumed at low to moderate levels [1-11].

The aims of this review are first to summarize the epidemiologic evidence supporting the association between ethanol ingestion and reductions in the likelihood of adverse cardiovascular events, even in patients with co-existing risk factors. As part of this objective, we will discuss the ideal quantities, drinking patterns, and types of alcoholic beverages that confer protective effects in the cardiovascular system. Second, we will review the findings of recent studies conducted in animal models and cell culture systems that are directed at uncovering the mechanisms that underlie the protective effects of antecedent ethanol ingestion, with an eye towards development of new therapeutic approaches that mimic the powerful protective effects of socially-relevant alcohol intake but minimize the negative psychosocial impact and pathologic effects of ethanol consumption.

Throughout this review, ethanol and alcohol are used interchangeably to denote ethyl alcohol. In addition, the term standard drink refers to one 5 ounce glass of wine, 12 ounces of beer, or 1.5 ounces of 80 proof spirits, each of which contain approximately 15-20 g of ethanol. Use of the phrase light to moderate drinking denotes ethanol consumption in the range of 1-2 standard drinks per day, while heavy alcohol consumption refers to intake of 3 or more standard drinks per day.

## 2. Epidemiologic evidence for the association between regular moderate ethanol intake and cardiovascular protection

Given the well-established pathologic effects of heavy drinking, wine lovers world-wide no doubt rejoiced when large-scale epidemiological studies first emerged showing that regular consumption of light to moderate amounts of alcoholic beverages, in particular red wine, was associated with a cardioprotective effect. One of the first studies showing a significant negative association between alcohol consumption and the risk of a subsequent first myocardial infarction that was well-controlled for cigarette smoking and other risk factors was published in 1974 [12]. Over the following 4 decades, numerous epidemiological studies including several meta-analyses (for example [13-51]) have consistently reported that an average alcohol consumption in the range of 0.5 to 2 standard drinks per day reduces coronary heart disease-related risks and ischemic stroke compared to non-drinkers.

Interestingly, initial work suggested that the protective effects associated with light to moderate intake of alcoholic beverages may be due to the polyphenols in grapes, wine, and dark beer. In particular, the stilbene resveratrol has been a major focus of research interest because this and other red wine constituent polyphenols exert powerful antioxidant actions, upregulate eNOS gene expression, enhance NO production and have been found to reduce morbidity and mortality due to cardiovascular disease [52-58]. In the human diet, red wine is one of the richest sources of polyphenols and moderate red wine drinkers consume these compounds at levels well above the population average [58]. Despite the demonstrated protective actions of resveratrol and flavonoids, discrimination between the effects of these red wine constituent polyphenols versus ethanol per se to induce protection associated with consumption of alcoholic beverages is controversial, in part owing to low levels of the former achieved in the blood following red wine ingestion [49]. Indeed, flavonoids and other wine polyphenols are extensively metabolized during absorption, resulting in formation of glucuronidated, sulfated, and methylated derivatives of the parent polyphenol, which have not been extensively evaluated for their protective effects [49,53,54]. As a consequence, the highest plasma concentrations achieved after ingestion of resveratrol-rich beverages by humans range between 1 and 10  $\mu$ M. These concentrations are lower than those typically evaluated in cell culture models and in animal studies. To further complicate the distinction between ethanol- and resveratrol-specific effects, the downstream molecular mechanisms so far identified for the two compounds show substantial overlap (reviewed in [59]). Nevertheless, important mechanistic distinctions exist, particularly with regard to the effects of resveratrol to induce the expression of several longevity genes, which have also been implicated in the cardioprotective effects of caloric restriction [52,53,58]. Some epidemiological studies have shown that consumption of red wine is more protective than other types of alcohol [29], whereas other studies failed to identify an additional advantage associated with red wine [38].

The health effects of ethanol are dependent on the amount of alcohol consumed and the pattern of intake. Nearly all epidemiologic studies report a J-shaped curve, whereby light to moderate ethanol consumption (1-2 standard drinks per day) exhibit less risk for adverse cardiovascular events and overall mortality than abstainers, while heavy drinkers (3-4 or more drinks per day) demonstrate increased risk [13-51]. As with liver injury induced by ethanol, this varies by sex, with women benefiting from 1 standard drink per day, whereas daily consumption of 1-2 drinks by males was associated with reduced total mortality [18,20]. On the other hand, increased mortality occurs in females with daily intake of 2 or more alcohol beverages and in men consuming more than 3 drinks per day.

The effects of light to moderate ethanol consumption appear to be most clearly related to cardiovascular benefits, with most studies reductions in risk for heart disease by 30-35% [31,39]. Regular alcohol consumption at low to moderate levels is associated with significant reductions in the incidence of myocardial infarction in both males and females, regardless of age in adults [61]. Importantly, this effect was noted in higher risk populations, including individuals with diabetes, hypertension, hypercholesterolemia, known heart disease, or who are overweight, as well as in cigarette smokers [13,16,19,23,24,28,34,43,47,49,51,59,61-73].

Cardiovascular disease risk is also lowered by moderate ethanol consumption in individuals adhering to healthy lifestyle behaviors. Indeed, it has been reported that men who exercised regularly for at least 30 min/day, abstained from smoking, adhered to a healthy diet, and maintained their body mass index at less than 25 kg/m<sup>2</sup> derived a health benefit from regular alcohol intake at moderate levels, demonstrating a 40-50% reduction in risk for myocardial infarction [37]. Moderate wine drinking also appears to strengthen the cardioprotective

effects of fish consumption, an observation that links two important components of the Mediterranean diet, namely omega-3 fatty acids and wine [74].

In addition to reducing the incidence and severity of myocardial infarction, low to moderate alcohol consumption is also associated with lower risk for ischemic stroke, dementia, congestive heart failure, peripheral artery disease, intestinal and hepatic I/R injury, and frequency of Raynaud's phenomenon [5,6,32,41,67,75-84]. Heart rate variability, a marker of autonomic imbalance, is also improved by consumption of alcoholic beverages, with wine intake demonstrating a stronger association with this effect than beer or spirits [84]. Reductions in C-reactive protein, fibrinogen, interleukin-6, and tumor necrosis factor alpha also occur with regular moderate alcohol consumption [82,85-88]. The aforementioned observations clearly indicate that the health benefits of alcohol consumption extend beyond the heart.

Although the concept that moderate intake of ethanol exerts protective effects in the cardiovascular system is now well accepted, important issues have been raised which challenge this premise. For example, it has been argued that uncontrolled confounding influences by lifestyle factors may play a role in the association between moderate alcohol intake and cardiovascular risk. Some work has led to the suggestion that individuals who regularly consume alcohol beverages exhibit healthier habits with regard to diet and/or exercise and enjoy superior sociodemographic factors, which could explain the reduced risk for ischemic myocardial disease [66,89-95]. However, systematic review and meta-analysis of interventional studies directed at the association between alcohol consumption and disease markers associated with risk for cardiovascular disease in adults without known cardiovascular disease argues against this supposition, as do the results of studies designed to better control for confounding sociodemographic factors and differences in dietary patterns and exercise adherence [18,20].

It has now become apparent that the pattern of drinking (which refers to the frequency and quantity of alcohol intake) exerts important effects on the association alcohol consumption and risk for cardiovascular disease. Binge drinking, which is defined as consumption of 3 or more alcoholic beverages within 1-2 hrs, is now recognized to increase the possibility for adverse cardiovascular risks [42,48,73]. Individuals pursuing this pattern of drinking often only consume alcohol one or two days during the week, so that their average intake over a given week may fall in the range of 1-2 drinks/day, an amount that exerts protection when ingested regularly each day. The fact that binge drinking exerts deleterious effects points to the importance of querying daily consumption versus number of drinks per week in evaluating data for the assessment of the health effects of ethanol consumption.

While the association between alcohol consumption and decreased cardiovascular risk is clear, it remains uncertain as to whether these correlative findings imply causation. However, a number of points regarding the aforementioned epidemiologic findings suggest that the association may indeed represent a cause-and-effect relationship [13-51]. First, there is a temporal relation between alcohol use and prevention of cardiovascular disease. Second, greater protection is observed with increasing ethanol dose over the protective range of alcohol intake. Third, the protective association between ethanol consumption and adverse cardiovascular events has been consistently observed in diverse patient populations and in both men and women. Fourth, the reduction in risk remains significant even after the influences of potential confounders such as cigarette smoking, diet, and exercise are factored into the analysis. Fifth, the association is specific for lowering rates of cardiovascular disease but does correlate with protection in other conditions such as cancer. Finally, the coupling of the aforementioned epidemiologic associations with the interventional

mechanistic studies discussed below, provides compelling support for the notion that ethanol intake may indeed confer protection against the deleterious effects of I/R.

### 3. Cardioprotective mechanisms induced by ethanol ingestion: direct influences on cardiomyocytes vs indirect extra-cardiac effects

Epidemiologic studies have also established associations which provide important clues regarding the mechanisms whereby ethanol intake may exert cardioprotective effects. Ethanol ingestion influences blood lipoprotein profiles, coagulation and fibrinolytic cascades, platelet aggregation, oxidative stress, insulin sensitivity, and endothelial function to reduce the risk of adverse cardiovascular events [13,22,25,26,35,40,42,45,50,70,72,85,96-110]. Use of experimental animal models and more simplified (reductionist) cell culture systems to examine the molecular mechanisms whereby ethanol confers cardioprotection has led to concept that ethanol may also directly induce cell survival programs in parenchymal cells that render them resistant to the deleterious effects of I/R. From this work, it has become apparent that alcohol consumption has beneficial effects on numerous targets that may be acting synergistically. As summarized in Figure 1, regular light-to-moderate alcohol consumption has direct effects on cardiomyocytes as well as vascular and extra-cardiac effects that positively influence strong risk factors of coronary heart disease.

With regard to indirect protective effects, chronic moderate alcohol consumption leads to beneficial alterations in blood lipid profile, platelet function, and fibrinolytic activity, all of which are directly linked to the risk of acute coronary events. For example, a 1999 meta-analysis of 42 experimental studies assessing the effects of moderate alcohol consumption on concentrations of high density lipoprotein cholesterol, apolipoprotein A I, fibrinogen, triglycerides, and other biological markers concluded that alcohol consumption over only 1-9 weeks leads to significant changes in blood lipids and clotting factors [46]. Based on published associations between these biomarkers and risk of coronary heart disease, the authors estimated that 30 g of alcohol a day would cause an estimated reduction of 24.7% in risk of coronary heart disease. Moreover, regular ethanol intake at light to moderate levels reduces oxidative stress, improves insulin sensitivity, and restores endothelium-dependent vasodilator responses in animal models and patients at risk for myocardial infarction, effects that persist for at least 24 hrs after ingestion [13,22,25,26,35,40,42,45,50,70,72,85,96-113].

Furthermore, regular light-to-moderate alcohol consumption also has beneficial effects with regard to diabetes and hypertension, two major risk factors of coronary artery disease. In epidemiological studies, alcohol consumption was associated with improved insulin sensitivity in diabetic patients after myocardial infarction [112] as well as in healthy non-diabetic women [11-113]. Moreover, moderate drinking also reduces the risk of newly developing type II diabetes [114]. In addition, regular light-to-moderate alcohol consumption also appears lower blood pressure. In young men, the lowest systolic blood pressure was found in subjects taking 1-3 drinks per day [115]. However, whether this is also true in other age groups or can be reproduced in patients who are already hypertensive patients is not yet known. Our understanding of the mechanisms underlying the effects of ethanol ingestion to improve insulin resistance and reduce blood pressure is also unclear.

Apart from improving the cardiovascular risk profile, ethanol also has direct effects on the coronary vasculature to increase myocardial blood flow. Ethanol-induced vasodilation occurs as a result of NO generation secondary to increased nitric oxide synthase (NOS) expression and activity and via activation of transient receptor potential vanilloid 1 (TRPV1) channels on perivascular sensory nerve terminals, which subsequently release the potent vasodilator calcitonin gene-related peptide (CGRP), thereby increasing coronary blood flow

[116-119]. Interestingly, polyphenols in red wine also increase NOS activity [120,121] and ingestion of red wine increases brachial artery blood flow in humans [122]. However, the increase in coronary blood flow following consumption was nearly identical to that produced by intake of an equivalent volume of ethanol per se (ingestion of which produced similar increments in blood alcohol), when evaluated in the same group of individuals [122]. These data suggest that the absorption of red wine polyphenols does not augment the vasodilator response induced by ethanol alone. Irrespective of the responsible mediator of coronary vasodilation induced by intake of alcoholic beverages, it is unlikely that this hyperemic effect is directly responsible for cardioprotection during I/R that occurs hours later, because this action of ethanol does not persist once the alcohol is metabolized. Indeed, the appearance of a protected phenotype induced by ethanol ingestion does not become manifest until the alcohol is removed from the experimental system [123-125]. Moreover, the concomitant presence of ethanol prevents the appearance of preconditioning induced by other interventions [123-126]. On the other hand, antecedent ethanol exposure enhances the beneficial actions of ischemic preconditioning in studies where sufficient time elapsed to allow the ingested alcohol to be metabolized [127].

In addition to the indirect beneficial effects associated with consumption of alcoholic beverages, ethanol ingestion increases the tolerance of cardiomyocytes to I/R by direct activation of cell survival programs. Indeed, acute and chronic alcohol administration in animal models has been found to decrease postischemic cell death and infarct size and to induce expression of known cardioprotective proteins such as NOS, heat shock proteins, and superoxide dismutase. Interestingly, antecedent ethanol ingestion appears to trigger activation of signaling cascades that are similar to those involved in another well-studied cardioprotective intervention, ischemic preconditioning (IPC). In IPC, a tissue is exposed to cycles of brief I/R prior to induction of prolonged period of coronary artery occlusion (referred to as index ischemia) and reperfusion [128]. Like antecedent ethanol ingestion, IPC markedly reduces myocardial I/R injury and does so in two temporal phases. The early phase (or classical IPC) arises within minutes after the bouts of preconditioning ischemia, relies on preexisting mediators, and disappears after 2-4 hrs. A second window of protection (SWOP, late phase or delayed IPC) reemerges 24 hrs later that is mediated by the expression of protective proteins and persists for up to 48-72 hrs [129]. In the next section, we will discuss in detail why and to what extent alcohol-induced cardioprotection resembles IPC.

#### **4. Molecular signaling mechanisms in ethanol-induced preconditioning of the heart**

Complementing and extending the results reported in epidemiological studies, work conducted in animal models has provided deeper insight into the molecular signaling mechanisms that underlie the infarct-sparing effects of antecedent ethanol exposure. Cardioprotective effects triggered by alcohol ingestion/exposure have been observed in various rodent models (mice, rats, guinea pigs, rabbits) as well as in dogs. Principally, two different approaches for ethanol exposure have been used: either chronic low-to-moderate alcohol consumption over at least a few weeks to simulate human drinking patterns or acute administration of a one-time dose of alcohol shortly before an ischemic insult.

The fact that both acute and chronic alcohol administration elicits protection against subsequent ischemic damage suggests that its underlying mechanisms may be akin to that elicited by ischemic preconditioning, which is also characterized by a first and a second window of protection. Not all signaling mediators and in particular the end-effector(s) of ischemic preconditioning have been identified to date. However, strong evidence has been presented for the critical roles of adenosine, bradykinin, opioids, protein kinase C, reactive oxygen species, the mitochondrial  $K_{ATP}$  channel, among others, in the first window (= early

phase or classic) preconditioning (reviewed in [80,130]). In second window (=late or delayed phase) preconditioning, signaling molecules such as ROS or NO, protein kinases such as protein kinase C, Akt, and MAPK, and transcription factors such as NF $\kappa$ B or STAT1/3 are recruited by the preconditioning stimulus leading to the transcription and synthesis of de novo proteins such as COX-2, heat shock protein, iNOS, aldehyde dehydrogenase, among others, that then protect the heart against ischemic damage via potential effects on the mitochondrial permeability transition pore (reviewed in [80,131]).

This wealth of information concerning the signaling cascades in early and late phase ischemic preconditioning provided an excellent starting point to unravel how alcohol intake can reduce myocardial infarct size. In animal models of acute alcohol-induced protection, a single low or moderate dose (in most studies equivalent to 0.5 - 2 drinks) given within 2 hours before the onset of ischemia/reperfusion led to a significant reduction in infarct size. This short-term protective effect could be abolished by pharmacological inhibition of protein kinase C (either with broad spectrum PKC inhibitors or agents that specifically target the PKC $\epsilon$  isoform), protein tyrosine kinases, and mitochondrial K<sub>ATP</sub> channels [132,133]. However, blockade of adenosine receptors or treatment with a free radical scavenger was ineffective in counteracting alcohol-induced protection [123]. Taken together, these data show that PKC $\epsilon$  activation plays a central role both in early ischemic preconditioning and in alcohol-induced protection. Furthermore, the mitochondrial K<sub>ATP</sub> channel appears to be important in either form of cardioprotection. However, upstream mechanisms at the receptor level appear to differ substantially, but this has not yet been characterized in great detail except for the contribution (or not) of adenosine receptors.

Mechanistic studies in animals that were fed alcohol at low-to-moderate doses over a long period of time before the ischemic insult (3-12 weeks) involve cardioprotective signaling elements that have been identified with known mediators of late phase IPC. In rat hearts, low-dose alcohol consumption over 3 weeks increased the expression levels of cardioprotective proteins such as hsp70, heme oxygenase-1, and manganese superoxide dismutase (MnSOD) [134]. Chronic alcohol feeding also increased the levels of activated PKC $\epsilon$  and Akt [135,136], and also upregulated eNOS expression and activity and NO generation [118,120]. Interestingly, the mitochondrial K<sub>ATP</sub> channel is also involved in the protective effects of chronic alcohol feeding [137-139]. Furthermore,  $\alpha$ 1-adrenoreceptors, adenosine A1-receptors, and phospholipase C have been implicated in the infarct-sparing effects associated with chronic ethanol intake in some species [140-142]. However, the temporal sequence of the signaling cascade invoked by chronic ethanol exposure has not been interrogated. Thus, it is unclear how these signaling elements are linked in a cascade that ultimately enables the appearance of a protected phenotype or whether species differences dictate how alcohol triggers cardioprotection.

Recently, another powerful cardioprotective mechanism has been described wherein tissues exposed to intermittent myocardial ischemia and reperfusion in the first few minutes after re-establishing reperfusion after prolonged ischemia significantly reduces the extent of myocardial tissue damage. Termed ischemic postconditioning or staccato reperfusion, this form of cardioprotection provides unequivocal evidence that reperfusion injury is distinct from that induced by ischemia per se. However, no studies have been conducted to date to suggest that alcohol may also exert a postconditioning-like protective effect if given at the onset of reperfusion. However, some of the signaling mediators identified in ischemic postconditioning such as adenosine receptors, PKC, and members of the RISK pathway (reviewed in [80,143]) are very likely to be activated by ethanol in the early reperfusion phase as well, suggesting that this approach should be evaluated.

## 5. Toxic ethanol metabolites and cardioprotection

After drinking an alcoholic beverage, the absorbed ethanol is metabolized predominantly by alcohol dehydrogenase to acetaldehyde. Acetaldehyde is far more reactive than ethanol itself, and is highly cytotoxic. Acetaldehyde is then further metabolized by mitochondrial aldehyde dehydrogenase to form acetate and NADH. Cardiac myocytes do not express alcohol dehydrogenase, but do express aldehyde dehydrogenase. Interestingly, ectopic expression of alcohol dehydrogenase in the hearts of transgenic mice exacerbates the toxic effects (e.g., depression of cardiac contractility) induced by acute alcohol administration [144,145]. In contrast, when the ability of the myocardium to detoxify aldehydes is enhanced by transgenic overexpression of aldehyde dehydrogenase-2 (ALDH2), the detrimental effects of chronic alcohol intake are ameliorated [146].

Recently, Chen and co-workers demonstrated that mitochondrial ALDH2 plays an important cardioprotective role [147]. These investigators discovered that in rat hearts that pretreatment either with ethanol or with a PKC $\epsilon$  activator before undergoing I/R resulted in the phosphorylation and thus activation of ALDH2, an effect that was inversely correlated to the extent of ischemic tissue damage. Using a high-throughput screening approach, this group identified small-molecule activators of aldehyde dehydrogenase-2 (designated Alda-1 and Alda-44), which when administered to rats before an ischemic event, greatly reduced infarct size [147,148]. These findings were independently confirmed by another group through use of a transgenic approach to overexpress ALDH2, which was associated with an infarct-sparing effect and improved contractile function during reperfusion [149].

The underlying mechanism of ALDH2-induced cardioprotection against ischemia is thought to be due to the resulting reduction in the formation of cytotoxic aldehydes such as 4-hydroxynonenal (4-HNE). Indeed, hearts subjected to I/R after ethanol pretreatment or IPC demonstrate significantly lower levels of 4-HNE adducts, indicating that this mechanism is shared by both cardioprotective interventions [150]. Furthermore, Chen and co-workers used an isoform-specific inhibitor to demonstrate that PKC $\epsilon$  is responsible for ischemia- as well as ethanol-induced activation of ALDH2 in rat hearts [147]. These findings were confirmed in a subsequent study which showed that ethanol failed to provide protection in PKC $\epsilon$  knockout mice whereas administration of an ALDH2 activator (Alda-44) effectively reduced infarct size in these animals [148]. These studies suggest that pretreatment of patients with ALDH2-activators before cardiac ischemia (such as coronary bypass surgery) may represent a promising new therapeutic approach to limit I/R injury. In particular, patients expressing inactive mutants of ALDH2, as often found in East Asian populations, may greatly benefit from such new treatments.

While the vast majority of publications demonstrate beneficial effects of moderate ethanol consumption, a few early studies reported that either acute or chronic ethanol administration failed to elicit cardioprotection [151-154]. However, the interpretation of these findings is clouded by the fact that ethanol was present during I/R in most of these studies, which was subsequently shown to prevent the appearance preconditioned phenotype in animal models [123-125]. In two of these studies, ethanol was administered acutely 10 min prior to I/R [151,153], which does not allow sufficient time for metabolic degradation to occur. In one of the chronic alcohol feeding studies that failed to show protection, significant blood alcohol levels were detected at the onset of the ischemia [31]. Subsequent work demonstrated that ethanol is cardioprotective, but only if ethanol is washed out of the tissues or sufficiently metabolized prior to the onset of I/R [123,124]. These findings have been extended to humans by Niccoli and co-workers [125] who found that ischemic preconditioning was abolished and the extent of ischemia worsened in patients given 40 g of alcohol shortly before coronary angioplasty. Although this is just a single study conducted in a small



number of patients, these data strongly suggest caution against providing ethanol to patients with acute chest pain or who are in danger of suffering an acute myocardial infarction within the next few hours.

While the mechanisms that contribute to the abrogating effects of the continued presence of ethanol on preconditioning are not clear, it has been suggested that alcohol dehydrogenase-catalyzed formation of acetaldehyde may interfere with removal of other harmful aldehydes such as 4-hydroxy-2-nonenol and malondialdehyde, both of which are generated during I/R by peroxidation of membrane lipids. Thus, insufficient ethanol washout or metabolism prior to I/R may lead to accumulation of cytotoxic aldehydes that worsen postischemic tissue injury by inactivating enzymes and disrupting structural proteins through the formation of protein adducts [123,124,155]. It has also been suggested that ethanol may activate protein kinase C isoforms that play opposing roles in I/R, with effects dependent on ethanol dose and timing for response evaluations [155].

However, such timing issues do not account for the discrepant findings reported in a third acute study wherein ethanol was provided long enough before the onset of ischemia to allow for its metabolism and removal of toxic by-products [153]. Furthermore, lack of washout was also not an issue in another study testing the effects of chronic alcohol feeding, since the hearts were isolated and perfused with ethanol-free buffer for the I/R protocol [27]. Therefore, these findings suggest that there might be other, as yet unidentified factors that influence the ability of ethanol to induce cardioprotection that may be uniquely explored in the I/R models used in these studies.

## 6. Protective effects of moderate ethanol ingestion in stroke

A considerable body of epidemiologic literature also associates moderate alcohol consumption with significantly reduced risks for cerebrovascular (ischemic) stroke, although at a less robust level than the reported for ischemic coronary disease [59]. In addition, ethanol ingestion has been shown to be effective in reducing dementia (induced by cerebral exposure to HIV-1 protein gp-120 or amyloid- $\beta$ , respectively), which may have an ischemic component [156-159], and stroke-induced injury in experimental animal models [160-165]. However, our understanding of the mechanisms whereby antecedent ethanol exposure limits cerebral I/R is less well-developed as compared to the cardiac literature.

One of the first studies in this area was reported in 2007 and indicated that ethanol ingestion 24 hrs prior to induction of cerebral I/R ameliorated protected the brain against postischemic delayed neuronal death, neuronal and dendritic degeneration, oxidative DNA damage, and glial cell activation, changes that were associated with improved behavioral deficits [164]. These beneficial effects were attenuated by coincident treatment with a NADPH oxidase inhibitor, indicating that antecedent ethanol ingestion at socially relevant levels induces neuroprotective effects in I/R by a mechanism that is triggered by ROS produced through NADPH oxidase [164]. In subsequent work from this group, it was shown that I/R-induced brain injury and inflammation (leukocyte rolling and adhesion) was attenuated not only by antecedent ethanol or but also by pretreatment with an activator of large conductance, Ca<sup>2+</sup>-activated potassium (BKCa) channels [165]. Since the protective effects of both forms of preconditioning were prevented by pretreatment with a BKCa channel inhibitor [165] or prior neutrophil depletion (unpublished observations), it appears that the neuroprotective effects of antecedent ethanol ingestion may also involve activation of these potassium channels and prevention of neutrophil-dependent neuronal injury. Finally, recent work has demonstrated that ethanol-induced neuroprotection involves activation of ionotropic glutamate receptors [166,167] in I/R, as depicted in Figure 2. Moderate ethanol ingestion also prevents the neurodegeneration induced by  $\beta$ -amyloid or other proinflammatory

peptides (eg, HIV-1 glycoprotein 120) that have been linked to dementia by a mechanism involving alcohol-induced activation of synaptic N-methyl-d-aspartate receptors [168-170] (Figure 2).

## 7. Adaptive cytoprotection in splanchnic organs after ethanol exposure

The ability of antecedent ethanol exposure to act as a mild irritant and induce the adaptive metamorphosis to a protected phenotype in the gastric mucosa was first described in 1979 by Robert and coworkers, a phenomenon referred to as adaptive cytoprotection in this literature [171-174]. Subsequent studies directed at examining the mechanisms whereby low concentrations of ethanol confer protection against noxious perturbations in the stomach have revealed an important role for prostaglandins, especially PGE<sub>2</sub> acting via EP1 receptors [171-180]. Other work has implicated a co-regulatory relationship between NO and prostaglandins, increased glutathione, activation of gastric dopamine or  $\beta$ 2-adrenergic receptors, improved mucosal blood flow, enhanced mucus secretion, stimulation of the internal enteric reflex, increased salivary secretions, prevention of intracellular calcium accumulation, and the formation of a protective layer of surface debris in the development of the defensive phenotype induced by ethanol exposure [176-179,181]. In addition to these mechanisms, antecedent ethanol exposure has been shown to limit the production of proinflammatory mediators such as leukotriene C<sub>4</sub>, an effect which may limit disturbances in microcirculatory function that otherwise would produce severe mucosal injury during subsequent exposure to necrotizing stimuli [176]. The latter observation led Dinda and coworkers [182] to propose that ethanol-induced adaptive cytoprotection is due to inhibition of neutrophil infiltration.

While most studies investigating the mechanisms of adaptive cytoprotection have involved assessment of mucosal damage in the stomach, several investigators have observed similar protective effects of antecedent ethanol exposure in the small intestine and liver. The mechanisms underlying ethanol-induced adaptive cytoprotection in the small bowel also appear to involve the production of prostaglandins [176,179,182]. However, it is important to emphasize that the intraluminal ethanol levels (20-100% ethanol) to which the small intestine was exposed in these studies, while relevant to the stomach, far exceeded those present in downstream segments of the gastrointestinal tract following oral intake. Indeed, the jejunum, ileum, and colon are exposed to ingested ethanol primarily via the bloodstream secondary to gastric absorption. Thus, the physiologic relevance of the mechanistic information obtained from studies employing luminal exposure, especially at a dose of 20-100% ethanol, is questionable. A more relevant ethanol exposure protocol was employed in a study examining hepatocellular protection and survival rates in rats exposed to liver I/R. In this report, rats received 40% ethanol at a dose of 5 g/kg by gavage, which produced increases in plasma ethanol that peaked at 180 mg/dl within two hours of ingestion, 24 hrs prior to induction of hepatic I/R. Postschemic liver injury, complement activation and deposition, and hepatic oxidant production were reduced and 14-day survival rates were remarkably improved by antecedent ethanol ingestion [78].

## 8. Ethanol exerts anti-inflammatory effects in I/R: cellular signaling mechanisms

Recognition of the fact that reperfusion can initiate a cascade of deleterious processes that exacerbate the tissue injury induced by ischemia has resulted in an intensive research effort directed at defining the cellular and molecular events that underlie I/R injury. Indeed, work conducted over the past 15 years has led to the development of the concept that oxidant-induced leukocyte/endothelial cell interactions are largely responsible for the microvascular dysfunction induced by reperfusion (reviewed in 80,183-186). Reactive oxygen species

generated by xanthine oxidase and other enzymes (eg, NAD(P)H oxidase) promote the formation of proinflammatory stimuli, modify the expression of adhesion molecules on the surface of leukocytes and endothelial cells, and reduce levels of the potent anti-adhesive agent nitric oxide. This latter effect is exacerbated by a postischemic decline in endothelial nitric oxide synthase activity and oxidation of soluble guanylyl cyclase (sGC), which serves to amplify the intense inflammatory responses elicited by I/R by reducing the bioavailability of NO and ability of downstream signaling elements to respond to this antiadhesive signaling molecule [166]. Coincident with these changes, perivascular cells (eg, macrophages, mast cells) become activated and release other inflammatory mediators (eg, TNF $\alpha$  and other cytokines, PAF, LTB $_4$ ). As a consequence of these events, leukocytes begin to form adhesive interactions with postcapillary venular endothelium. Platelets also play an important role in the adhesion of leukocytes to the postischemic microvasculature. The activated leukocytes emigrate into the tissues, inducing microvascular barrier dysfunction via release of oxidants and hydrolytic enzymes. In addition to these changes, leukocytes also contribute to postischemic nutritive perfusion failure (fewer perfused capillaries, ie, capillary no-reflow), endothelium-dependent vasoregulatory dysfunction in arterioles, and parenchymal cell dysfunction. Thus, leukocyte-endothelial cell adhesive interactions, which precipitate the development of arteriolar, capillary, and postcapillary venular dysfunction in the microcirculation, are among the earliest signs of tissue dysfunction and injury elicited by I/R [80,183-186].

A growing body of evidence indicates that antecedent ethanol ingestion abrogates P-selectin expression, leukocyte-platelet/endothelial cell interactions, oxidant generation, capillary no-reflow, mitochondrial dysfunction, release of proinflammatory mediators, impaired arteriolar endothelium-dependent vasoregulation, and microvascular barrier disruption induced by I/R 24 hrs later [187-204]. As depicted in Figure 3, these beneficial actions are instigated by ethanol-induced activation of xanthine oxidase and NADPH oxidase, which produce superoxide, coincident with adenosine-A $_2$  receptor-dependent activation of the  $\alpha$ 2 isoform of AMPK, which phosphorylates and activates eNOS [164,189,191,199-201]. Concomitant production of superoxide and NO leads to the formation of reactive nitrogen oxide species (such as peroxynitrite), which may stimulate cysteine  $\gamma$ -lyase and/or cystathionine  $\beta$  synthase to produce hydrogen sulfide (H $_2$ S). RNOS and H $_2$ S activate K $_{ATP}$  channels on capsaicin-sensitive sensory neurons, sensitizing them to ethanol and rendering them resistant to down-regulation, thereby provoking release of CGRP [190,195,206-209]. CGRP activates cystic fibrosis transmembrane regulator (CFTR) function by a cAMP/PKA-dependent mechanism, thereby enhancing platelet NO (effects that were absent in CFTR $\Delta$ 506 mice and inhibited by CFTR $_{inh1-72}$ , unpublished observations), and also large conductance, calcium-activated potassium channels (BKCa), which may induce the expression of heme oxygenase-1 (HO-1) during I/R 24 hrs later [163,204,205] (Figure 3). The reaction products of HO-1 (CO, biliverdin and secondarily derived bilirubin) act to abrogate adhesion molecule expression and thus prevent postischemic leukocyte/endothelial cell adhesive interactions. This preserves endothelial barrier function, which is now protected from neutrophil-dependent injury mechanisms during I/R. Although inhibitor studies ruled out a role for IKCa and SKCa in the effect of H $_2$ S to induce tolerance to ischemia, other pharmacologic activators of these channels can produce preconditioning [203], providing another potential avenue for exploration in the development of anti-ischemia agents. Ethanol-induced signaling also upregulates HO-1 by an ARE/Nrf2-dependent mechanism [210], which may serve to protect soluble guanylyl cyclase from redox inactivation in reperfused tissues [211] (Figure 3).

In addition to its influence on vascular endothelial cells, moderate ethanol consumption also exerts anti-inflammatory effects in human adipose tissue. While promoting the expression of adiponectin, a fat-derived adipokine that exerts powerful anti-inflammatory effects, ethanol

or wine ingestion also reduced the release of inflammatory cytokines and pro-inflammatory mediators from human adipose tissue under basal conditions and following exposure to lipopolysaccharides [13,212,213].

Importantly, the aforementioned anti-inflammatory effects are manifest in animals ingesting ethanol at volumes that produce peak plasma concentrations (40-50 mg/dl blood) that occur in humans consuming 1-2 alcoholic beverages, but are absent in mice consuming ethanol at levels that raise blood alcohol to 300 mg/dl. Moreover, the postischemic anti-inflammatory effects induced by moderate ethanol consumption persist in mice with co-existing risk factors, as discussed above. The latter observation is exceedingly important because numerous studies suggest that the effectiveness of IPC as an anti-ischemic intervention is impaired or eliminated when tested in hypertensive, hypercholesterolemic or diabetic animal models (reviewed in 80). This may explain why IPC does not always appear to produce beneficial effects in humans, who typically present with co-morbid risk factors. When taken together, these latter two findings will no doubt fuel a concerted effort to define why the presence of risk factors impairs IPC, with a focus on developing new therapeutic strategies that circumvent the cellular processes that limit the effectiveness of this intervention in the presence of co-morbidities. Examination of the cellular mechanisms by which antecedent ethanol ingestion confers protection may provide important clues in this regard.

## 9. Summary

Abundant epidemiologic evidence and the results interventional mechanistic studies conducted in animal models and cell culture systems strongly support the notion that antecedent ethanol exposure at moderate levels confers protective cardiovascular effects. Mechanistic interrogation of the signaling pathways invoked by antecedent ethanol ingestion may point the way towards development of new therapeutic approaches that mimic the powerful protective effects of socially relevant alcohol intake to limit I/R injury, but minimize the negative psychosocial impact and pathologic outcomes that also accompany consumption of alcoholic beverages. Indeed, preconditioning can be induced by pharmacologic administration of agents identified as triggers (eg, adenosine and CGRP receptor agonists, NO donors), downstream mediators (BKCa channel agonists), and effectors (eg, agents such as hemin, which upregulates HO-1 expression) in lieu of ethanol. Finally, exciting new findings indicate that intake of alcoholic beverages at moderate levels enhances neovascularization and capillary densities in ischemic tissues of diabetic mice by enhanced mobilization of circulating endothelial progenitor cells to these regions [214]. These and other provocative findings should provide the impetus for continued exploration of novel pathways to invoke cell survival programs that limit ischemic injury.

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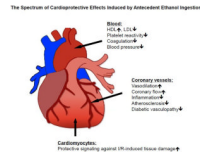
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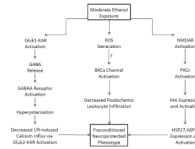
### Highlights

- Epidemiologic evidence that ethanol ingestion is cardioprotective is summarized.
- Molecular signaling mechanisms invoked by antecedent ethanol consumption.
- Protective actions of ethanol ingestion in stroke and peripheral vascular disease.
- Anti-inflammatory signaling mechanisms invoked by ethanol ingestion.



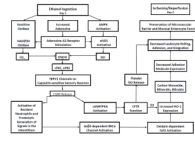


**Figure 1.** Antecedent ethanol ingestion protects the myocardium by direct effects to activate cell survival signaling programs in cardiac myocytes and indirectly via affects to reduce risk factors such as hypertension and atherosclerosis, decrease platelet reactivity and coagulation, and modify coronary vessel function.



**Figure 2.**

Mechanisms for induction of a preconditioned neuroprotected phenotype by ethanol in stroke. Ethanol exposure activates ionotropic glutamate receptors of the kainite Gluk1 receptor (Gluk1-KAR) subtype to release gamma-aminobutyric acid (GABA) from intraneuronal synaptic terminals, which subsequently activates postsynaptic GABA<sub>A</sub> receptors (left arm of figure). The ensuing influx of chloride ions through activated GABA<sub>A</sub> receptors hyperpolarizes the membrane, thereby decreasing I/R-induced calcium influx through kainite Gluk2 receptors (Gluk2-KAR) via an effect to suppress assembly of Gluk2, postsynaptic density protein-95, and mixed-lineage kinase 3 signaling module induced by ischemia, which in turn inhibits the c-Jun N-terminal kinase 3 (JNK3) apoptotic pathway. Gluk1-KAR activation also decreases tyrosine phosphorylation of synaptic N-methyl-d-aspartate receptors (NMDAR), which may also contribute to neuroprotection. The central arm illustrates the effect of ingestion of moderate levels of ethanol to produce an anti-inflammatory phenotype such that postischemic leukocyte/endothelial interactions are suppressed. The attenuation of neutrophil-dependent neuronal injury and cell death appears to be initiated by the effect of ethanol at moderate levels to invoke the formation of reactive oxygen species (ROS) and activation of large conductance, calcium-activated potassium (BK<sub>Ca</sub>) channels, cytoprotective events that may be mechanistically linked since oxidants can activate these potassium channels. Interestingly, NMDAR are thought to be initial pro-survival sensors for ethanol, engendering protection against dementia-inducing neuroinflammatory proteins (right arm of figure) via activation of protein kinase C epsilon (PKC $\epsilon$ ) and focal adhesion kinase (FAK), which in turn induce elevations in protective heat shock proteins (HSP). Since age-dependent cognitive decline and/or dementia are thought to have an ischemic component, the disparate neuroprotective roles of NMDA receptor activation versus inhibition in dementia vs ischemic stroke implies dramatically different receptor regulation of downstream signaling elements in these conditions.



**Figure 3.**

Ethanol ingestion 24 hrs at moderate levels prior to the onset of ischemia/reperfusion induces the appearance of an anti-inflammatory phenotype such that postcapillary venules fail to express endothelial cell adhesion molecules, thereby attenuating leukocyte recruitment to ischemic tissues. Development of this protected phenotype is instigated by the effect of ethanol to trigger the generation of superoxide ( $O_2^-$ ) secondary to activation of xanthine oxidase and NADPH oxidase and endothelial nitric oxide synthase (eNOS)-dependent formation of nitric oxide (NO) in the vascular wall. Superoxide interacts with NO to form reactive nitrogen oxide species such as peroxynitrate (ONOO<sup>-</sup>), which together with  $O_2^-$  and NO, activate transient receptor potential vanilloid 1 (TRPV1) channels on capsaicin-sensitive sensory neurons. This initiates release of calcitonin gene-related peptide (CGRP) which produces downstream signals by at least two mechanisms. First, CGRP activates cAMP/protein kinase A-dependent signaling to stimulate cystic fibrosis transmembrane regulator (CFTR) function in endothelial cells and/or platelets. While it is not clear how this chloride channel couples to downstream signaling elements to effect the anti-inflammatory phenotype, it likely links with expression of pro-survival protein expression (such as heme oxygenase-1 or HO-1). Another possibility relates to CFTR-stimulated production of the antiadhesive gasotransmitter NO by platelets. CGRP also activates resident neutrophils, thereby causing release of proteases that sever extracellular matrix molecules to expose cryptic sites within or release bioactive cleavage products from these interstitial structures. This results in  $\alpha v \beta 3$  integrin-dependent activation of large conductance, calcium-activated potassium ( $BK_{Ca}$ ) channels, which results in oxidant-dependent Nrf2 activation. These events culminate in increased expression and activity of HO-1. This enzyme catalyzes the rate limiting step in heme metabolism, forming the anti-adhesive molecule carbon monoxide, as well as the powerful anti-oxidants biliverdin and bilirubin. These reaction products decrease endothelial cell adhesion molecule expression, thereby reducing leukocyte rolling, adhesion and emigration and preventing leukocyte-dependent reperfusion injury.