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Moderate Ethanol Preconditioning of Rat Brain Cultures Engenders Neuroprotection Against Dementia-Inducing Neuroinflammatory Proteins: Possible Signaling Mechanisms

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Abstract There is no question that chronic alcohol (ethanol) abuse, a leading worldwide problem, causes neuronal dysfunction and brain damage. However, various epidemiologic studies in recent years have indicated that in comparisons with abstainers or never-drinkers, light/moderate alcohol consumers have lower risks of age-dependent cognitive decline and/or dementia, including Alzheimer's disease (AD). Such reduced risks have been variously attributed to favorable circulatory and/or cerebrovascular effects of moderate ethanol intake, but they could also involve ethanol "preconditioning" phenomena in brain glia and neurons. Here we summarize our experimental studies showing that moderate ethanol preconditioning (MEP; 20–30 mM ethanol) of rat brain cultures prevents neurodegeneration due to β-amyloid, an important protein implicated in AD, and to other neuroinflammatory proteins such as gp120, the human immunodeficiency virus 1 envelope protein linked to AIDS dementia. The MEP neuroprotection is associated with suppression of neurotoxic protein-evoked initial increases in $[Ca^{+2}]_i$ and proinflammatory mediators—e.g., superoxide anion, arachidonic acid, and glutamate. Applying a sensor → transducer → effector model to MEP, we find that onset of neuroprotection correlates temporally with elevations in "effector" heat shock proteins (HSP70, HSP27, and phospho-HSP27). The effector status of HSPs is supported by the fact that inhibiting HSP elevations due to MEP largely restores gp120-induced superoxide potentiation and subsequent neurotoxicity. As upstream mediators, synaptic *N*-methyl-d-aspartate receptors may be initial prosurvival sensors of ethanol, and protein kinase C epsilon and focal adhesion kinase are likely transducers during MEP that are essential for protective HSP elevations. Regarding human consumption, we speculate that moderate ethanol intake might counter incipient cognitive deterioration during advanced aging or AD by exerting preconditioning-like suppression of ongoing neuroinflammation related to amyloidogenic protein accumulation.

Keyword Moderate ethanol preconditioning · Alzheimer's disease · Neuroinflammatory proteins

Abbreviations

CNS	central nervous system
MEP	moderate ethanol preconditioning
HEC	hippocampal–entorhinal cortical
HIV	human immunodeficiency virus
PKC	protein kinase C

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FAK	focal adhesion kinase
NMDAR	*N*-methyl-d-aspartate receptor
A1R	adenosine$_1$ receptor

Introduction

The phenomenon of preconditioning tissues or cells to bring about a cytoprotective state is well established [1, 2]. For example, brief ischemia that can engender ischemic tolerance has been studied extensively in the heart and, to a lesser extent, the brain. Such ischemic as well as hyperthermic preconditioning to achieve brain neuroprotection apparently was only first recognized about two decades ago [3, 4]. Preconditioning-dependent protection by alcohol (ethanol) against ischemic insult of the heart is supported by a range of studies [5, 6], such that the phenomenon is considered a likely component of cardioprotection in moderate alcohol consumers [7]. However, despite accumulating evidence for a relationship between light-moderate ethanol consumption and reduced risk of dementia or cognitive decline, recently summarized in two meta-analyses [8, 9], there is limited experimental study of ethanol and brain preconditioning mechanisms. One notable in vivo exception is an acute ethanol/cerebral ischemia study with gerbils [10]. These authors found that antecedent ethanol treatment significantly attenuated postischemic neuronal injury and behavioral deficit. Their pharmacologic results indicated that reactive oxygen species (ROS) derived from brain nicotinamide adenine dinucleotide phosphate oxidase were critical signaling molecules inaugurating entry into a neuroprotective preconditioning state.

To examine ethanol preconditioning and its potential neuroprotective mechanisms against neuroinflammatory proteins—technically, cross-preconditioning—to date, we have used an ex vivo model of organotypic slice cultures of the maturing rat hippocampal–entorhinal cortex (HEC) complex [11], which incorporates two brain regions significantly impacted in dementias including Alzheimer's disease (AD) and AIDS dementia, and in vitro cultures of rat cerebellar cells containing 20%–30% neurons and 70%–80% glia ("mixed" cultures). While ROS may well have roles, our results have shed light on other mediators that include *N*-methyl-d-aspartate (NMDA) receptors, protein kinases, and heat shock proteins (HSPs) (Fig. 1).

Moderate Ethanol Preconditioning Neuroprotection Against Human Immunodeficiency Virus 1 Proteins or β-Amyloid Species

Several neurotoxic human immunodeficiency virus 1 (HIV-1) proteins are believed to underlie AIDS encephalitis and dementia [12]. The most potent experimentally, envelope glycoprotein 120 (gp120), causes neuronal degeneration and apoptosis in organotypic hippocampal slices and other primary brain cultures at picomolar concentrations [13, 14]. Two other retroviral proteins, gp41 (anchor protein for gp120) and Tat, a *trans*-activating factor, also display significant neurotoxicity, but nanomolar or higher concentrations are needed in cultures. We examined the effect of moderate ethanol pretreatment (or preconditioning; MEP)—20–30 mM, typically a subneurotoxic ethanol concentration range—on neurodegeneration due to 1–2 days of gp120$_{IIIB}$ exposure in the above cultures, and observed nearly complete neuroprotection [15, 16]. The duration of ethanol preconditioning required for statistically significant neuroprotection prior to addition of gp120 was >4 days [16], but the importance of this timeframe was not appreciated until HSPs were examined during MEP (vide infra).

Neuroinflammatory processes originating from gp120$_{IIIB}$ interactions with glial chemokine receptors (especially CXCR4 and CCR5) are thought to trigger neurotoxic cascades [12]. We found that MEP effectively blunted or suppressed some of these processes or events that were usually stimulated soon (within seconds to minutes) after gp120 addition to the HEC slice cultures—specifically, increases in $[Ca^{+2}]_i$, arachidonic acid release, tissue superoxide levels, and extracellular glutamate concentrations [16]. Interestingly, MEP did not significantly suppress increases in nitric oxide induced by gp120. Nevertheless, Western blot analyses indicated that protein nitrosylation in HEC slice cultures was modestly increased by gp120 and likewise normalized by MEP (N. Achille, unpublished data). Thus, the formation from superoxide and nitric oxide of the transient nitrosylating agent, peroxynitrite anion, is possibly regulated by the former free radical rather than the latter. To our knowledge, this is the first evidence of direct induction of brain protein nitrosylation by gp120, but whether nitrosylated proteins have a role in its neurodegenerative effects remains unanswered.

Neuroprotection in HEC slices by MEP was also demonstrable with other neurotoxins that employ glial activation as an important part of neuronal killing, such as the HIV-1 proteins gp41 and Tat, and the AD-related peptide, β-amyloid (1-42) [17]. More recently, rat cerebellar cultures have been used in studies of MEP protective mechanisms versus the neurotoxic β-amyloid (25-35) peptide fragment (see below).

Neuroprotective Mechanisms Initiated by Ethanol Preconditioning: HSP as Possible Effectors

Considerable research indicates that HSPs are putative neuroprotective "effectors" induced by ischemic preconditioning [1, 18]. The HSP molecules implicated in various precondi-

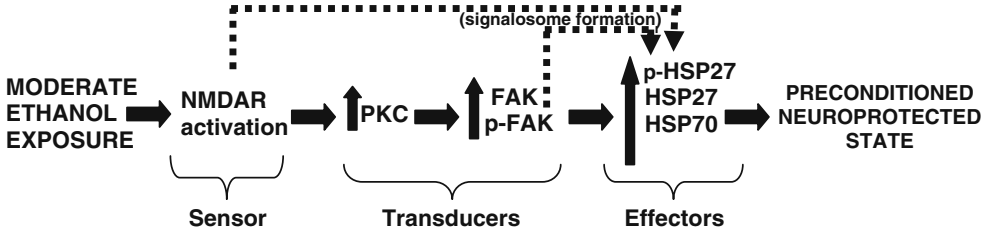

Fig. 1 Scheme for moderate (20–30 mM) ethanol preconditioning (MEP) indicating a possible sequence of signal transduction events, based on current evidence, that leads to a neuroprotected state. *Dashed arrows* imply that NMDAR and p-FAK entities ultimately may constitute a signalosome complex with effector HSP (especially p-HSP27). *NMDAR* *N*-methyl-d-aspartate receptor, *PKC* protein kinase C, *FAK* focal adhesion kinase, *p-FAK* tyrosine-phosphorylated focal adhesion kinase, *HSP* heat shock protein, *p-HSP27* tyrosine-phosphorylated heat shock protein 27

tioning modalities include but are not limited to HSP27, HSP32, HSP60, HSP70, GRP78, and HSP90. It is germane, therefore, that we observed significant elevations of HSP70 and HSP27 in HEC slices after 6 days of moderate ethanol exposure—but not at 4 days—which correlates with the emergence of neuroprotection against gp120, as mentioned above [19]. Furthermore, phospho-HSP27 (p-HSP27), which is known to colocalize with cytoskeletal elements including actin [20], was increased both in the HEC slices and cerebellar cultures after MEP for 6 days (Sivaswamy et al., submitted for publication). Interestingly, initial immunochemical localization experiments indicate that the apparent induction of HSP70 is primarily neuronal, whereas HSP27 augmentation is more diffuse and extensively astroglial. It is worth noting that the MEP-dependent HSP changes were selective, since three other HSP molecules—HSP32, HSP90, and GRP78—were not increased at any timepoint. To establish HSP essentiality, we suppressed HSP70 and HSP27 elevations and examined MEP neuroprotection. Indeed, cotreatment of HEC slice cultures throughout 6 days of MEP with quercetin (5 μM), a general HSP induction inhibitor, blocked HSP potentiation by ~80% and negated the neuroprotective outcome. Furthermore, preliminary siRNA knockdown experiments indicated that HSP27 and HSP70 were both important for full neuroprotection. Overall, the results to date have suggested that the two HSP molecules are critical effector components in MEP neuroprotection against toxins such as gp120, since inhibiting their potentiation abrogated neuroprotection while concomitantly restoring the superoxide increases [19]. Further experiments are underway to confirm that the HSP elevations are due to selective induction of HSP messages.

Neuroprotective Mechanisms Initiated by Ethanol Preconditioning: Protein Kinase C and Focal Adhesion Kinase as Possible Transducer Kinases

To examine signal transduction events potentially upstream of and integral to the observed HSP elevations, protein kinase C (PKC) activity and changes in PKC isoform expression were examined in HEC slices and cerebellar mixed cultures at various times throughout moderate (30 mM) ethanol exposure. PKC activity was moderately but significantly increased ~15% at 2 days and was ~40% above control activity at day 6 of ethanol. The increased activity at the 2-day timepoint appeared related not to higher PKC levels (Western immunoblots) but to increased membrane translocation of PKCε (and PKCα). However, after 4 and 6 days of ethanol, we observed relatively large increases in PKCε expression (~200%), accompanied by modest increases in PKCα and PKCδ. Furthermore, with cerebellar cultures, coexposure with a pan-PKC inhibitor (GF109203X) during the latter 3 days of 6-day MEP significantly suppressed HSP27 elevations and completely blocked the rise in HSP70 and p-HSP27 [21].

Focal adhesion kinase (FAK) and Y397-phosphorylated (activated) FAK are pivotal nonreceptor protein kinase forms associated with extracellular matrix-dependent integrin signaling and cytoskeletal focal adhesions. Studies with ischemic-preconditioned myocytes have linked FAK with cytoprotective elevations in HSP27 and HSP70 [22]. The relatively specific induction in rat brain slices and cerebellar cultures of these two HSP molecules by MEP stimulated our exploration of FAK's role in the resulting neuroprotective state. Accordingly, Western blot analyses of FAK and Y397 p-FAK in our cultures revealed that MEP significantly increased the levels of both forms at the 4- to 6-day treatment intervals [21].

There is evidence with other tissues that PKC activity can regulate actin dynamics [23], possibly via FAK and related integrin signaling entities. Using dominant negative PKC isoforms, it was ascertained that PKCε activity was at least indirectly responsible for increases in FAK and p-FAK as well as in the HSP effectors during MEP. However, PKCα did not seem essential to neuroprotective MEP signaling via FAK. Knowing now that FAK signaling was downstream of PKCε, we then examined the effect of dominant negative FAK, termed *FRNK*, on the HSP effectors and neuroprotection in the cerebellar cultures. As

we hypothesized, adenovirus-directed FRNK expression greatly reduced MEP-mediated increases in HSP27, p-HSP27, and HSP70. Consistent with this finding, FRNK overexpression during MEP reestablished the neurotoxicity of β-amyloid (25-35), thus confirming the novel transduction role of FAK in MEP's neuroprotective mechanism (S. Sivaswamy et al., in preparation). The scheme in Fig. 1 indicates the possible role of p-FAK, evidently downstream of PKCε activity, in signal transduction triggered by neuroprotective preconditioning.

Neuroprotective Mechanisms Initiated by Ethanol Preconditioning: Synaptic NMDA Receptors as Potential Sensors

Studies in other preconditioning models have elucidated the involvement of initial cellular sensors that instigate subsequent transducer mechanisms [1, 2]. Such sensors have included glutamate receptors (especially *N*-methyl-d-aspartate receptors or NMDAR), adenosine receptors (notably A_1R), bradykinin receptors, and perhaps other receptors coupled to $G_{i/o}$ proteins. MEP experiments with mixed cerebellar cultures using the receptor antagonists, memantine or AP-5 for NMDAR, 2,8-cyclopentyl-1,3-dipropylxanthine for A_1R, and pertussis toxin for $G_{i/o}$ protein-coupled receptors, demonstrated that activation of NMDAR—but not of A_1R or other $G_{i/o}$ protein-coupled receptors—is important during the first several days of moderate ethanol exposure [24]. Consistent with this result, immunoblot analyses revealed modest but significant upregulation of NMDAR subunits (NR1, NR2B, NR2C) during early MEP that could underlie activation of this receptor; moreover, memantine blocked up-regulation of the obligate NR1. These results are consistent with those of Maler et al. [25], who found similar changes with 50 mM ethanol ± memantine exposure for 5 days in hippocampal cultures—although their interpretations were concerned with ethanol neurotoxicity, not neuroprotection. That NMDAR activation is indeed a functional upstream sensor linked to transducers and resulting neuroprotection in our MEP studies is supported by the facts that the presence of memantine during the first 2 days of MEP antagonizes later p-FAK increases and restores neurotoxicity due to β-amyloid (Mitchell et al., in preparation).

In view of emerging evidence that activation of synaptic pools of NMDAR promotes prosurvival, viability mechanisms (as opposed to extrasynaptic NMDAR that are possibly linked to better-known excitotoxic, proinflammatory pathways triggering Ca^{+2} dyshomeostasis) [26, 27], we examined moderate ethanol's effect on a protein indicator of synaptic NMDAR enrichment and localization, postsynaptic density-95 (PSD-95). At 2 days of ethanol exposure, there occurred significant increases in PSD-95, as well as in another marker relatively specific to synaptic NMDAR, tyrosine 1472-phosphorylated NR2B (Y1472-NR2B) [24]. More recently, results have indicated that by an early stage of MEP, elevations in Src tyrosine kinase and pyk-2, a FAK homolog, which could be catalyzing Y1472-NR2B formation and aiding synaptic NMDAR enrichment, are significant events (R. Mitchell et al., in preparation). In addition, an antioxidant enzyme, peroxiredoxin-II, appeared to be up-regulated after 6 days of MEP and suppressed by memantine's copresence—a finding consonant with facts that synaptic NMDAR activity regulates antioxidant defenses [28]. Applying immunocytochemistry and deconvolution microscopy, whether MEP does promote synaptic colocalization of Y1472-NR2B, PSD-95, and the tyrosine kinases mentioned above is being investigated.

Summary and Conclusions

In concert with what is known about alcohol abuse and the brain, subchronic exposure of adult rats as well as organotypic brain slice cultures to high ethanol concentrations (80–120 mM, blood levels observed in chronic alcoholics) combined with withdrawal episodes causes neurodegeneration [29-31]. However, treatment of the slices or other mixed primary cultures with moderate, subneurotoxic ethanol concentrations effectively preconditions the cellular population to withstand various neurotoxic insults—in our studies, dementia-associated proteins such as β-amyloid and gp120. Activation of NMDAR, increases in PKC isoforms, and elevations in neuroprotective HSP effectors due to ethanol preconditioning have been reported in other more studied models of brain preconditioning. Also, as recently reviewed by Nagy [32], NMDAR modification/regulation by ethanol has been extensively investigated, albeit mostly with high, often neurotoxic ethanol levels that would be expected to target both synaptic and nonsynaptic NMDAR subunits. However, we posit that FAK and p-FAK are novel critical players in the mechanism of increased brain HSP and subsequent neuroprotection. Accordingly, Fig. 1 adheres to a temporal "sensor → transducer → effector" model for these observed changes leading to a neuroprotective state. One implication of this scheme, which has had some literature support [22, 33], is that the structural integrity of the (neuronal) cytoskeleton may be a surveillance system in transmitting (neuroprotective) stress signals. Further experiments will explore the possibility that synaptic NMDAR, p-FAK, p-HSP27, HSP70, and several of the above kinases ultimately constitute a distinct signalosome complex stabilizing (or stabilized by) the cytoskeleton.

Returning to humans, what significance might these brain mechanisms induced by moderate ethanol exposure have in real life? As noted [8, 9], cross-sectional or case-control studies from several countries provide evidence that moderate ethanol consumption is linked to a reduction in the risks of age-related cognitive decline and/or dementia, including AD. However, it should be mentioned that some studies report no cognitive improvement associated with moderate consumption [34-36]. While the ethanol levels that induce neuroprotection in our brain culture experiments are somewhat more elevated (albeit shorter in duration) than in periodic, human consumption, it is nevertheless conceivable that low-to-moderate, stable ethanol intake over years exerts aspects of the "preconditioning" effects on glia and neurons that we have observed, leading to dampening of the incipient neuroinflammatory processes associated with brain aging.

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