Ethanol-Induced Contractions in Cerebral Arteries
Role of Tyrosine and Mitogen-Activated Protein Kinases

Zhi-wei Yang, MD, PhD; Jun Wang, MD; Tao Zheng, MD; Bella T. Altura, PhD; Burton M. Altura, PhD

Background and Purpose—The relationship between alcohol consumption and stroke appears complex; moderate ingestion is associated with reduced stroke risk, while heavy intake is associated with increased stroke risk. Ethanol has been shown both experimentally and epidemiologically to induce hemorrhagic and ischemic strokes, which are associated with cerebral vasoconstriction. Ethanol is known to induce contraction in isolated cerebral arteries and intact microvessels from diverse mammalian animals. The relationships between ethanol-induced contractions in cerebral arteries, intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)), tyrosine kinases (including the src family), and mitogen-activated protein kinases (MAPK) were investigated in the present study.

Methods—Cerebral arterial muscle tension and [Ca\(^{2+}\)]\(_i\) were quantified by an isometric contraction technique and direct visualization of Ca\(^{2+}\) in single cells.

Results—Ethanol induces concentration-dependent contractions in intact canine basilar arteries, which are attenuated significantly by pretreatment of the arteries with low concentrations of an antagonist of protein tyrosine kinases (genistein); an src homology 2 (SH2) domain inhibitor peptide; a highly specific antagonist of p38 MAPK (SB-203580); a potent, selective antagonist of MEK1/MEK2 (U0126); and a selective antagonist of mitogen-activated protein kinase kinase (MAPKK) (PD-98059). IC\(_{50}\) levels obtained for these 5 antagonists are consistent with reported \(K_i\) values for these tyrosine kinase, MAPK, and MAPKK antagonists. Ethanol induces transient and sustained increases in [Ca\(^{2+}\)]\(_i\) in primary single smooth muscle cells from canine basilar arteries, which are markedly attenuated in the presence of genistein, an SH2 domain inhibitor peptide, SB-203580, U0126, and PD-98059. Several specific antagonists of known endogenously formed vasoconstrictors do not inhibit or attenuate either the ethanol-induced contractions or the elevation of [Ca\(^{2+}\)]\(_i\).

Conclusions—The present study suggests that activation of protein tyrosine kinases (including the src family) and MAPK appear to play important roles in the ethanol-induced contractions and the elevation of [Ca\(^{2+}\)]\(_i\) in smooth muscle cells from canine basilar arteries. The results could be used to suggest that selective antagonists of protein tyrosine kinases and MAPK may be useful both prophylactically and therapeutically in alcohol-induced strokes. (Stroke. 2001;32:249-257.)

Key Words: alcohol ■ basilar artery ■ calcium ■ dogs ■ protein kinases ■ tyrosine kinases

Although it is believed that moderate alcohol intake, especially moderate wine intake, may exert cardioprotective action, numerous studies over the past 2 decades have affirmed that the risk for developing cerebral vascular diseases, such as stroke, clearly increases with increasing alcohol consumption. Recent observations of the in situ cerebral microcirculation and a variety of isolated mammalian cerebral arteries indicate that acute treatment with ethanol produces prolonged constrictions in cerebral blood vessels, suggesting that such vasoconstrictive actions of ethanol are involved in the hypoxic, ischemic, and hemorrhagic actions of alcohol in the brain. Support for the latter concept derives from recent studies of ethanol-induced stroke in intact animals.
ogy 2 (SH2) domain-containing adaptor proteins and play a role in agonist-induced activation of Ras. Mitogen-activated protein kinase kinase (MAPKK), or MEK (a cytosolic nonreceptor protein kinase), is in the family of tyrosine kinases. Mitogen-activated protein kinase proteins (MAPK), substrates of MAPKK, known as extracellular signal-regulated kinases, serve to relay, amplify, and integrate diverse signals, thus allowing a cell to coordinate a physiological response. Several observations raise the possibility that activation of tyrosine kinase might be involved in ethanol-induced constriction in gastric smooth muscle, and ethanol might induce a stimulation of MAPK in aortic smooth muscle. However, there is no direct evidence that tyrosine kinases or MAPK are associated with ethanol-induced cerebral vasocostriction.

The mechanisms underlying ethanol-induced contraction of cerebral vascular smooth muscle and stroke remain less well understood. With these points in mind, we designed our present study to test the hypothesis that the contractile effects of ethanol on cerebral arteries are due, in large measure, to activation of protein tyrosine kinases, recruitment of SH2 domain adaptor proteins, and activation of MAPK and that recruitment of these enzyme pathways collectively results in modulation of [Ca\(^{2+}\)].

**Materials and Methods**

**General Procedures**

Rings of canine basilar arteries were obtained from male mongrel dogs (weight, 18 to 22 kg), after administration of pentobarbital sodium anesthesia (40 mg/kg IV), as described previously, and placed in normal Krebs-Ringer bicarbonate solution at pH 7.4 containing the following (in mmol/L): NaCl 118, KCl 4.7, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 1.2, CaCl\(_2\) 2.5, dextrose 10, and NaHCO\(_3\) 25. The rings were 3 to 4 mm in length. The endothelium was denuded as described previously, and the denuded endothelium rings precontracted with prostaglandin F\(_2\alpha\) (PGF\(_2\alpha\)) failed to relax >90% to maximal concentrations of substance P. The segments were mounted on stainless steel pins under 2 g resting tension in isolated organ baths, attached to force transducers (Grass model FT 03), and connected to Grass model 7 polygraphs. The organ baths, containing normal Krebs-Ringer bicarbonate solution, were gassed continuously with 95% O\(_2\) and 5% CO\(_2\) and warmed to 37°C (pH 7.4). Tissues were allowed to equilibrate for at least 90 minutes before data collection. At the beginning of an experiment, rings were exposed for 30 to 45 minutes to 80 mmol/L KCl, and this was repeated every 30 to 45 minutes, until responses were stable (2 to 3 times). When tissues were pretreated by various drugs, the drug was applied for at least 15 minutes before the concentration-response curves were obtained. All of the animal experimental procedures were approved by our institutional animal care and use committee.

**Intracellular Ca\(^{2+}\) Measurement**

Primary smooth muscle cells from canine basilar arteries for image analysis experiments were seeded on glass coverslips (12 mm diameter; approximately 1x10\(^5\) cells per coverslip) and used 2 to 3 days after seeding, as described in the literature. Monolayers of the smooth muscle cells, grown on the coverslips, were loaded with 2.0 μmol/L fura 2-AM and 0.12% pluronic acid F-127 (60 minutes, 37°C), and the experimental procedures for [Ca\(^{2+}\)]\(_i\) measurements were performed as described previously using fura 2-AM. The resulting images were then used to calculate [Ca\(^{2+}\)]\(_i\) in smooth muscle cells. [Ca\(^{2+}\)]\(_i\) was calculated according to the following equation:

\[
[Ca^{2+}]_i = K_d \times B \times (R - R_{	ext{max}})/(R_{	ext{max}} - R)
\]

A K\(_d\) of 224 nmol/L was used for the fura 2/Ca\(^{2+}\) complex. B is the ratio of fluorescence intensity of fura 2 to Ca\(^{2+}\):fura 2 complex excited at 380 nm. Particular care was taken to minimize photobleaching of the dye. Experiments were performed in total darkness, and exposure to excitation light was <2 seconds in all experiments.

**Drugs**

The following pharmacological agents were purchased from Sigma Chemical Co: daidzein, EGTA, genistein, naloxone HCl, and prananolol HCl. Atporphine sulfate was bought from MANN Research Laboratory Inc. U0126 was purchased from Promega Co. SB-203580 was bought from Torcis Cookson Inc. Cimetidine HCl and diphenhydramine HCl were received from Smith Kline & French Laboratories Ltd. Dimethyl sulf oxide, PD-98059, and an SH2 domain inhibitor peptide were purchased from CALBIOCHEM Corporation. Phentolamine methanesulfonate was purchased from CIBA Pharmaceutical Company. Methysergide maleate was received from Sandoz Pharmaceuticals. Indomethacin was received as a gift from Merck Inc. All other organic and inorganic chemicals were obtained from Fisher Scientific and were of the highest purity.

**Calculations and Statistical Analysis**

The contractile response (g), percentage of maximal KCl-induced contraction, and [Ca\(^{2+}\)], were expressed as mean±SEM. Statistical evaluation of the results was performed by analysis by the Newman-Keuls test and ANOVA with Scheffe’s contrast test. The results were considered significant at a value of P<0.05.

**Results**

Ethanol-Induced Vasoconstrictions Are Inhibited by a Tyrosine Kinase Antagonist and an SH2 Domain Inhibitor

To our knowledge, 20 mmol/L ethanol should be considered moderate ethanol intake, because it can be found in the blood of most humans after oral ingestion of only 1 to 2 oz of ethanol, and 90 to 200 mmol/L ethanol should be considered heavy to very heavy ethanol intake; 88 mmol/L ethanol is known to be found in the blood of humans with ethanol-induced stroke like episodes. Therefore, we used a concentration range of 20 to 200 mmol/L ethanol in the present study. Although not shown, intact versus endothelium-denuded cerebral arterial rings failed to manifest any significant differences in contractile concentration-response curves to ethanol (n=36; P>0.05). Only intact vessel rings were used in the present study. As shown in Figure 1A, ethanol produces a rapid contractile response (rapid component) that is followed by a prolonged, slightly diminished stable increase in vessel tension (stable component) in intact canine basilar arterial rings. Pretreatment of intact canine basilar arteries with genistein (an antagonist of protein tyrosine kinases) or an SH2 domain inhibitor peptide, but not daidzein, an inactive homologue of genistein, significantly attenuates ethanol-induced contractions (both rapid and stable components) in a concentration-dependent manner (Figure 1A and 1B). The IC\(_{50}\) values for genistein and an SH2 domain inhibitor for such inhibition of the contractions are 5.6±0.23×10\(^{-3}\) and 0.73±0.06×10\(^{-6}\) mmol/L (Figure 1B), respectively. Mean values for vasoconstrictions induced by varying concentrations of ethanol, in the absence (control) and presence of genistein or an SH2 domain inhibitor, are shown in Figure 1C.
Tyrosine Kinase Antagonist and SH2 Domain Inhibitor Attenuate Ethanol-Induced Elevations in [Ca$^{2+}$]

Figure 2A demonstrates that ethanol produces a rapid [Ca$^{2+}$], peak, followed by a steady state [Ca$^{2+}$], plateau in primary cultured single smooth muscle cells obtained from canine basilar arteries. Preincubation of primary cultured smooth muscle cells from intact canine basilar arteries with genistein or an SH2 domain inhibitor, but not daidzein, for 15 minutes effectively prevents both the transient elevation in [Ca$^{2+}$], and the additional sustained rise of [Ca$^{2+}$] induced by ethanol (Figure 2A). Lower steady states and a loss of the rapid, peak increment in [Ca$^{2+}$], are now seen. Such inhibitory effects of these 2 antagonists display concentration-dependent effects (Figure 2B). The concentrations producing 50% of the maximal inhibitory effects (IC$_{50}$ values) for genistein and an SH2 domain inhibitor, for such attenuation of the increases in [Ca$^{2+}$], are $5.2 \pm 0.16 \times 10^{-5}$ and $0.68 \pm 0.04 \times 10^{-6}$ mol/L, respectively (Figure 2B), which is consistent with the reduced vasoconstriction induced by ethanol in the presence of these antagonists under the same conditions. Mean peak [Ca$^{2+}$] values obtained under different concentrations of ethanol, in the absence and presence of these antagonists, are shown in Figure 2C.

Ethanol-Induced Contractions Are Inhibited by MAPK and MAPKK Antagonists

Figure 3A and 3B illustrates that the presence of PD-98059 (a selective antagonist of MAPKK$^{25}$), SB-203580 (a highly specific antagonist of p38 MAPK$^{26}$), or U0126 (a potent, selective antagonist of MEK1/MEK2$^{27}$) attenuates contractile responses (both rapid and stable components) of intact canine basilar arteries to ethanol in a concentration-dependent manner. The calculated IC$_{50}$ values for PD-98059, SB-203580, and U0126 for such inhibition of the contractions are $8.1 \pm 0.2 \times 10^{-6}$, $1.62 \pm 0.2 \times 10^{-6}$, and $0.58 \pm 0.09 \times 10^{-6}$ mol/L, respectively (Figure 3B). Mean values for varying concentrations of ethanol-induced contractions, in the absence (control) and presence of PD-98059, SB-203580, or U0126, are shown in Figure 3C.

MAPK and MAPKK Antagonists Attenuate Ethanol-Induced Elevations in [Ca$^{2+}$]

Figure 4A demonstrates that preincubation of the cells with PD-98059, SB-203580, or U0126 for 15 minutes effectively inhibits both the transient [Ca$^{2+}$], peak and the sustained plateau of [Ca$^{2+}$], induced by ethanol (to lower steady states) in basilar arterial smooth muscle cells. The inhibitory effects of these 3 antagonists show clear concentration-dependent effects (Figure 4B). The calculated IC$_{50}$ values for PD-98059, SB-203580, and U0126 for such attenuation of the increases in [Ca$^{2+}$], are $7.8 \pm 0.3 \times 10^{-5}$, $1.53 \pm 0.09 \times 10^{-5}$, and $0.46 \pm 0.08 \times 10^{-5}$ mol/L,
Figure 2. Concentration-dependent inhibitory effects of tyrosine kinase antagonists and an SH2 domain inhibitor on \([Ca^{2+}]_i\) changes in single smooth muscle cells, obtained from intact canine basilar arteries, induced by varying concentrations of ethanol. For A, vertical bar denotes \([Ca^{2+}]_i\) (nmol/L); horizontal bar, time (min). For A and C, concentrations of genistein, daidzein, and an SH2 domain inhibitor used herein are \(6.0 \times 10^{-5}\), \(6.0 \times 10^{-5}\), and \(10^{-6}\) mol/L, respectively; preincubation time of these antagonists was 15 minutes. For B and C, each point represents the peak value and mean ± SE expressed as \([Ca^{2+}]_i\) (nmol/L). n = 12 to 14; for C, \#P < 0.05, *P < 0.01, **P < 0.001.

Figure 3. Contractile responses of intact canine basilar arteries to varying concentrations of ethanol are modified by MAPK and MAPKK antagonists. For A, vertical bar denotes tension (g); horizontal bar, time (minutes). For A and C, concentrations of PD-98059, SB-203580, and U0126 used herein are \(10^{-5}\), \(2 \times 10^{-6}\), and \(10^{-6}\) mol/L, respectively; preincubation time of these antagonists was 15 minutes. For B and C, each point represents the peak value and mean ± SE expressed as tension (g). n = 7; for C, \#P < 0.05, *P < 0.01, **P < 0.001.
respectively, which is in close agreement with the IC50 values found for the reduced vasoconstriction produced by ethanol in the presence of these antagonists under the same conditions. Mean values for varying concentrations of ethanol-induced elevation in [Ca2+]i in the absence (control) and presence of PD-98059, SB-203580, or U0126, are shown in Figure 4C.

Effects of Tyrosine Kinase and MAPK Antagonists and a SH2 Domain Inhibitor on Ethanol- and PGF2α-Precontracted Canine Basilar Arterial Segments

After achieving full contractile responses of isolated intact canine basilar arterial rings to 200 mmol/L ethanol, we noted that administration of 6.0 × 10^-6 mol/L genistein, 10^-5 mol/L PD-98059, 2.0 × 10^-6 mol/L SB-203580, 10^-6 mol/L U0126, but not 6.0 × 10^-6 mol/L daidzein, led to a reduction of the ethanol contractions to 60%–70% of the initial level (Figures 5A and 6A). The administration of the same concentrations of genistein, an SH2 domain inhibitor, PD-98059, SB-203580, and U0126, but not daidzein, also brought about significant relaxation in PGF2α-precontracted isolated cerebral arteries (Figures 5B and 6B). However, the effects of these antagonists on ethanol-precontracted cerebral arterial segments (Figures 5A and 6A) are much stronger than those of equipotent PGF2α-precontracted segments (P<0.05, Figures 5B and 6B).

Failure of Several Specific Pharmacological Antagonists to Attenuate or Interfere With Ethanol-Induced Vasoconstrictions

Incubation of canine basilar arterial rings with a variety of pharmacological antagonists (ie, diphenhydramine [10^-6 mol/L], cimetidine [10^-5 mol/L], phenotlamine [10^-6 mol/L], methysergide [10^-6 mol/L], propranolol [10^-5 mol/L], atropine [10^-6 mol/L], naloxone [10^-5 mol/L], and indomethacin [10^-5 mol/L]) for 15 minutes, before stimulation with ethanol, failed to either inhibit or attenuate cerebrovasospasms induced by the alcohol (n=6 each; data not shown). Likewise, these antagonists failed to attenuate the rises in [Ca2+]i produced by ethanol (data not shown).

Discussion

The present investigation aimed to determine whether activation of tyrosine kinase (including the src family) and MAPK is associated with contractile responses of cerebral arteries to ethanol, thereby giving us some insight into the potential contribution of these 2 cellular signaling pathways to ethanol-induced cerebral vasoconstriction.

Previous studies have demonstrated that tyrosine kinase (including the src family) activation is important in several vasoconstrictor- and epidermal growth factor–induced vascular contractions.14,28–30 These studies provide the foundation that, in addition to their mitogenic activities, tyrosine kinase(s) and src itself may play some important roles in agonist-induced smooth muscle contraction. However, little knowledge is currently available concerning the actions of tyrosine kinases in ethanol-induced cerebral vasoconstriction. As the present study has demonstrated, the ability of genistein (an antagonist of protein tyrosine kinase) and an SH2 domain
inhibitor peptide to impair ethanol-induced contractions in canine basilar arterial segments implicates the involvement of tyrosine kinase activation (phosphorylation, including src) in the cerebrovascular contractile responses of cerebral smooth muscle to ethanol. We used daidzein, a structurally similar but inactive form of genistein, in the present study as a control agent to test the selectivity of tyrosine kinase antagonists, especially genistein. The calculated IC₅₀ values reported herein for genistein and the SH2 domain inhibitor peptide are in a range similar to the reported Kᵢ values of genistein for protein tyrosine kinase 24 and SH2-SH3/phosphoprotein interaction.³¹ Our present findings are well supported by a recent study in which the tyrosine kinase antagonists, genistein and tyrphostin-47, were reported to inhibit the contractile action of ethanol on guinea pig gastric smooth muscle, although these data were not obtained from cerebral blood vessels.¹⁷
The involvement of tyrosine kinase, including the src family, is reinforced by the present findings that genistein and an SH2 domain inhibitor peptide suppress both the ethanol-induced transient and sustained increments in [Ca\(^{2+}\)], in single canine basilar smooth muscle cells at calculated IC\(_{50}\) values, which are in good agreement with those of ethanol-induced arterial contractions under the same conditions and are consistent with previously published \(K_i\) values for these 2 antagonists.\(^{24,31}\) These data suggest that contraction of the cerebral arteries to ethanol may be mediated, at least partially, by an elevation in [Ca\(^{2+}\)], in canine basilar arterial smooth muscle cells modulated by activation of tyrosine kinase, including src. This conclusion gains strong support from several lines of recent experimental findings: (1) platelet-derived growth factor BB elicits Ca\(^{2+}\) dependent mechanism;\(^{32}\) (2) genistein can inhibit the activity of L-type Ca\(^{2+}\) channels in vascular smooth muscle cells from rat portal vein;\(^{33}\) (3) serotonin-evoked Ca\(^{2+}\) release from the sarcoplasmic reticulum in vascular smooth muscle cells is blocked by genistein;\(^{44}\) and (4) tyrosine phosphorylation by both nonreceptor and receptor tyrosine kinases could be an important mechanism by which voltage-operated channels are regulated in vascular muscle.\(^{35,36}\)

Several recent studies indicate that MAPK plays important roles in diverse vasoconstrictor-induced contraction in rat cerebral arteries, rat aorta, and guinea pig gastric longitudinal smooth muscle.\(^{28,30,37}\) These previous findings implicate the MAPK pathway in modulation of vascular smooth muscle contractility and, as a tyrosine kinase, MAPKKK might be a logical candidate to be activated by ethanol stimulation. An important observation presented herein is that PD-98059 (a specific MAPK antagonist), SB-203580 (a highly specific antagonist of p38 MAPK), and U0126 (a potent and selective antagonist of MEK1/MEK2) produce significant concentration-dependent attenuation of ethanol-induced contractions in intact canine basilar arteries. The calculated IC\(_{50}\) values for PD-98059, SB-203580, and U0126 are consistent with the reported \(K_i\) values for these 3 antagonists.\(^{25–27}\) These results suggest that activations of both MAPKK and MAPK pathways in cerebral arterial smooth muscle cells play important roles in these ethanol-induced contractile responses. This concept derives strong support from a very recent study that demonstrates that concentrations of ethanol (17 to 170 mmol/L) similar to those used in the present study could induce a dose-dependent stimulation of p44/p42 MAPKs in rat aortic smooth muscle cells.\(^{18}\)

It has been shown previously that MAPK is involved in angiotensin and low [Mg\(^{2+}\)]\(_r\)-induced contraction and elevation of [Ca\(^{2+}\)]\(_r\)-stimulated [Ca\(^{2+}\)], increases in rat cerebral and aortic smooth muscle cells.\(^{16,37–39}\) This may be a major pathway by which ethanol leads to increments of [Ca\(^{2+}\)], in smooth muscle cells from canine basilar arteries and activates MAPKKK and MAPK in the smooth muscle cells. In this context, our present findings indicate that PD-98059, SB-203580, and U0126 significantly inhibit the ethanol-induced concomitant rise in [Ca\(^{2+}\)], in single cells from canine basilar smooth muscle at calculated IC\(_{50}\) values, which are in close agreement with those of the ethanol-induced arterial contrac-

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Alcohol drinking and abstinence are both part of our social culture. The epidemiological relationships between alcohol drinking and stroke appear to be “U-shaped” and are a frequent source of controversy in both popular press and medical/scientific literature. Light-to-moderate alcohol consumption was recently reported to reduce overall stroke risk and risk of ischemic stroke in male physicians, stimulating ongoing interest and cautions about alcohol’s potential health benefits. Notably, moderate alcohol consumption (frequently defined as 2 drinks per day) decreases ischemic stroke risk in elderly, multiethnic subjects of both sexes after adjustment for cardiac disease, hypertension, smoking, and diabetes. Ethanol’s dose-restrictive, antioxidant activity may be important in these results (for review, see Hillbom). Whether ethanol ingestion increases or decreases overall stroke or stroke subtype risk appears to be dependent not only on oral dose and formulation but also on drinking pattern (regular versus binge drinking) and the presence of alcohol-induced hypertension and proembolic changes, including thromboxane-mediated platelet activation. Nevertheless, numerous studies indicate that heavy recent alcohol drinking is an independent risk factor for all major subtypes of stroke. In animals, the majority of work agrees that acute alcohol induces cerebral arterial constriction. It is to these observations that the present study of Yang et al offers pathophysiological insight and new information about the signaling pathways of ethanol in intact canine basilar artery and primary vascular smooth muscle cells. These data carefully characterize dose-response relationships within ethanol concentrations that would be expected to be pathological, not protective, in human stroke. Ethanol-induced vasoconstrictor mechanisms are not limited to tyrosine kinase and mitogen-activated protein kinase in large cerebral vessels (for review, see Altura and Altura). Future work will be tasked with determining cross-talk among controllers of calcium cycling in the cerebrovascular contractile apparatus.

Patricia D. Hurn, PhD, Guest Editor
Department of Anesthesiology/Critical Care Medicine
Johns Hopkins Medical Institutions
Johns Hopkins Hospital
Baltimore, Maryland

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