Afoabazole Modulates Neuronal Response to Ischemia and Acidosis via Activation of σ-1 Receptors

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Received April 8, 2011; accepted June 28, 2011

ABSTRACT

Afoabazole is an anxiolytic medication that has been previously shown to be neuroprotective both in vitro and in vivo. However, the mechanism(s) by which afoabazole can enhance neuronal survival remain poorly understood. Experiments were carried out to determine whether afoabazole can decrease intracellular calcium overload associated with ischemia and acidosis and whether the effects of afoabazole are mediated via interaction of the compound with σ receptors. Fluorometric Ca2+ imaging was used to resolve how application of afoabazole affects intracellular Ca2+ handling in cortical neurons. Application of afoabazole significantly depressed, in a concentration-dependent and reversible manner, the intracellular Ca2+ induced by acidosis. The effects of afoabazole on ischemia- and acidosis-induced intracellular Ca2+ dysregulation were inhibited by preincubating the neurons in the irreversible, pan-selective σ-receptor antagonist, metaphet. Moreover, the effects of afoabazole on intracellular Ca2+ increases triggered by acidosis and ischemia were blocked by the selective σ-1-receptor antagonists, BD 1063 and BD 1047, respectively. Experiments examining the effects of afoabazole on neuronal survival in response to ischemia showed that afoabazole was neuroprotective. Taken together, these data suggest that afoabazole regulates intracellular Ca2+ overload during ischemia and acidosis via activation of σ-1 receptors. This mechanism is probably responsible for afoabazole-mediated neuroprotection.

Introduction

Afoabazole (5-ethoxy-2-[2-(morpholino)-ethylthio]benzimidazole) is an anxiolytic drug that was approved for clinical use in Russia in 2005. In addition to the anxiolytic properties, afoabazole has been shown to decrease neuronal death in vitro in response to oxidative stress and glutamate excitotoxicity (Zenina et al., 2005). In vivo, afoabazole has been shown to be neuroprotective in a rat cerebral hemorrhage model and a rat prefrontal cortex photoinfarcion model (Galavet et al., 2005; Seredenin et al., 2008). Several mechanisms have been proposed to mediate these neuroprotective effects of afoabazole, including inhibition of nitric-oxide synthase and increased superoxide dismutase activity (Seredenin et al., 2008). In addition, it has also been suggested that modulation of GABAergic transmission and regulation of brain-derived neurotrophic factor may contribute to the beneficial effects of afoabazole in various pathophysiological models (Zenina et al., 2005; Szanyi et al., 2007; Seredenin et al., 2008). However, the molecular mechanisms underlying afoabazole-mediated neuroprotection remain poorly understood.

The chemical structure of afoabazole shares the pharmacophore structure with the σ-1-receptor ligand, (+)-pentazocine (Walker et al., 1990), which motivated a recent study on the interaction of afoabazole with these receptors (Seredenin et al., 2009). Afoabazole was found to displace [3H](+)-pentazo- cine in a Jurkat cells assay with an IC50 of approximately 10 μM (Seredenin et al., 2009). Moreover, afoabazole was found to promote mobilization of σ-1 receptors to the plasma membrane in HT-22 cells, also suggesting an afoabazole-σ-1-receptor interaction (Seredenin et al., 2009). However, it has not been established whether afoabazole functions as an agonist or an antagonist at σ-1 receptors. Furthermore, the role of σ-1 receptors in afoabazole-mediated neuroprotection remains unknown.

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σ-1 Receptors have shown to modulate various processes that contribute to neuroprotection during pathophysiological states, in particular, neuronal ischemia. For example, σ-1-receptor activation has been shown to decrease intracellular Ca$^{2+}$ overload produced by both in vitro ischemia and acidosis (Katnik et al., 2006; Herrera et al., 2008). This inhibition of intracellular Ca$^{2+}$ dysregulation is mediated via the inhibition of multiple ion channel types, including the acid-sensing ion channel 1a (ASIC1a) (Katnik et al., 2006; Herrera et al., 2008). The inhibition of these multiple pathways probably accounts for the fact that stimulation of σ receptors in vivo decreases neuronal injury in a rat model of ischemic stroke, even when these receptors are activated 24 h after onset of ischemia (Ajlom et al., 2006). Given the fact that afobazole shares the pharmacophore structure with the σ-1 agonist (+)-pentazocine, these receptors represent a putative target for afobazole-mediated neuroprotection.

Experiments were carried out to determine the effects of afobazole on intracellular Ca$^{2+}$ overload produced by ischemia and acidosis in cortical neurons. Furthermore, the role of σ receptors on afobazole regulation of neuronal responses to these pathophysiological states was examined. Afobazole was found to significantly depress the intracellular Ca$^{2+}$ dysregulation triggered by ischemia and acidosis. Moreover, afobazole was found to enhance neuronal survival during in vitro ischemia. Inhibition of σ receptors via the pan-selective antagonist metapiracet decreased the effectiveness of afobazole in both the ischemia and acidosis assays. Experiments using the σ-1-selective antagonists BD 1047 and BD 1063 showed that this receptor is specifically involved in the effects of afobazole in neurons.

Materials and Methods

Primary Rat Cortical Neuron Preparation. Primary cortical neurons were isolated from embryonic (embryonic day 18) rats and cultured as described previously by our laboratory (Katnik et al., 2006). All procedures were done in accordance with the regulations of the University of South Florida Institutional Animal Care and Use Committee. Neurons grown in culture for 10 to 21 days were used for this study.

Calcium Imaging Measurements. The effects of afobazole on ischemia- and acidosis-evoked changes in intracellular Ca$^{2+}$ concentrations in neurons were examined using fluorescent imaging techniques with fura-2 as the indicator (Katnik et al., 2006). Cells plated on coverslips were incubated for 1 h at room temperature in Neurobasal (Invitrogen, Carlsbad, CA) medium supplemented with B27 (Invitrogen) and 0.5 mM l-glutamine or in physiological saline solution (PSS) consisting of 140 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl$_2$, 1.0 mM MgCl$_2$, 20 mM glucose, and 25 mM HEPES, pH to 7.4 with NaOH. Both solutions contained 3 μM ace2toxymethyl ester (Invitrogen). Afobazole was generously provided by Masterclone (Moscow, Russia).

Data Analysis. Analyses of measured intracellular Ca$^{2+}$ and membrane current responses were conducted using Clampfit 9 (Molecular Devices, Sunnyvale, CA). Imaging data files collected with SlideBook 4.02 (Intelligent Imaging Innovations, Inc., Denver, CO) were converted to a text format and imported into Clampfit for subsequent analysis. Statistical analysis was conducted using SigmaPlot 9 and SigmaStat 3 software (Systat Software, Inc., San Jose, CA). Statistical differences were determined using unpaired $t$ tests for within group and between group experiments, and were considered significant if $p < 0.05$. For multiple group comparisons, either a one-way or a two-way analysis of variance, with or without repeat measures, was used as appropriate. When significant differences were determined with an analysis of variance, post hoc analysis was conducted using a Tukey’s test to determine differences between individual groups. For the generation of concentration response curves, data were best fit using a single-site Langmuir-Hill equation.

Results

Experiments were carried out to determine whether application of afobazole affects elevations in [Ca$^{2+}$], that occur in response to acidosis in rat cortical neurons. Focal application of PSS solution at pH 6.0 produced robust elevations in [Ca$^{2+}$], in the neurons (Fig. 1A), consistent with previous reports (Herrera et al., 2008). In the presence of afobazole, the increase in [Ca$^{2+}$], was reduced (Fig. 1A), and this inhibition was reversible (data not shown). The concentration-response relationship for afobazole-mediated inhibition of acidosis-evoked [Ca$^{2+}$], increases was determined using identical experiments. Afobazole decreased the elevations in [Ca$^{2+}$], elicited by acidosis in a concentration-dependent manner. Figure 1B shows a plot of the mean increase in [Ca$^{2+}$], evoked by acidosis as a function of afobazole concentration, with the data normalized to control (no afobazole). A fit to the data using a single-site Langmuir-Hill equation indicates that afobazole inhibits the [Ca$^{2+}$] elevations with a half-maximal concentration of 164 μM.

The elevations of [Ca$^{2+}$], evoked by protons in cortical neurons are attributed to activation of ASIC, but these channels themselves account for only a small portion of the increase in [Ca$^{2+}$], observed during acidosis (Herrera et al.,...
Much of the proton-evoked increases in \( [\text{Ca}^{2+}]_i \) is attributed to the opening of channels downstream of ASIC (Herrera et al., 2008). To determine whether afobazole is blocking ASIC or only downstream effector targets of these channels, patch-clamp electrophysiological recordings were carried out. Figure 2A shows proton-evoked membrane currents recorded in the absence and presence of 300 \( \mu \text{M} \) afobazole from a neuron held at a membrane potential of \( -70 \text{ mV} \). Application of protons evoked a rapid inward current, which was reduced by afobazole. In a second neuron, application of psalmotoxin 1 (PcTx) and inhibitor of homomeric ASIC1a channels (Chen et al., 2005) decreased proton-evoked current amplitude, and coapplication of afobazole (300 \( \mu \text{M} \)) with PcTx resulted in further reduction in current amplitude (Fig. 2B). In identical experiments, afobazole inhibited proton-evoked current by 18 ± 2 and 50 ± 11% in the absence and presence of PcTx, respectively. Thus, afobazole has greater effects on the PcTx-insensitive component of the proton-evoked current, which is probably mediated by ASIC1a/ASIC2a-heteromeric channels (Escoubas et al., 2000; Askwith et al., 2004).

Given that afobazole shares pharmacophore structure with the \( \sigma-1 \) agonist pentazocine, experiments were conducted to determine whether \( \sigma \) receptors are involved in the inhibition of acidosis-evoked increases in \( [\text{Ca}^{2+}]_i \), by afobazole. For these experiments, we used the irreversible \( \sigma \) receptor antagonist metaphit, which blocks both \( \sigma-1 \) and \( \sigma-2 \) receptors. Figure 3A shows representative traces of \( [\text{Ca}^{2+}]_i \), as a function of time recorded from two cells during acidosis. In the cell preincubated in 50 \( \mu \text{M} \) metaphit for 15 min before the recordings...
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Fig. 3. Inhibition of α receptors blocks afobazole-mediated suppression of proton-evoked increases in [Ca\(^{2+}\)], A, representative traces of [Ca\(^{2+}\)], as a function of time recorded from two different neurons during acidosis in the absence (Control) and presence of 300 μM afobazole, without (PSS, left traces) and with 15 min preincubation in 50 μM metaphor (Metaphit, right traces). B, bar graphs of mean (±S.E.M.) increases in peak [Ca\(^{2+}\)], (∆[Ca\(^{2+}\)] \(\text{Peak}\)) or net [Ca\(^{2+}\)], (Area, area under the trace) elicited by acidosis in the absence (Control) and presence of afobazole when cells were preincubated in normal PSS \(n=163\) or PSS containing 50 μM metaphor for 15 min \(n=135\). C, bar graph of percentage inhibition of peak (Peak) and net (Area) acidosis-evoked increases in [Ca\(^{2+}\)], produced when cells were treated with 300 μM afobazole (Afob) or with afobazole after 15-min preincubation in metaphor (Afob + MET). Asterisks in B and C denote significant differences between Control and afobazole groups within PSS and metaphor, respectively (\(p<0.001\)). Pound symbols in C denote significant difference between PSS and metaphor within the afobazole group (\(p<0.001\)).

Fig. 4. The α-1-receptor antagonist BD 1063 inhibits afobazole-mediated suppression of proton-evoked increases in [Ca\(^{2+}\)], A, representative traces of [Ca\(^{2+}\)], as a function of time recorded from a neuron during acidosis in the absence (Control) and presence of 300 μM afobazole (Afob) and 300 μM afobazole + 10 nM BD 1063 (Afob + BD 1063). Application of BD 1063 was initiated 5 min before application of afobazole and maintained throughout afobazole treatment. B, bar graphs of mean (±S.E.M.) increases in peak [Ca\(^{2+}\)], (∆[Ca\(^{2+}\)] \(\text{Peak}\)) or net [Ca\(^{2+}\)], (Area, area under the trace) elicited by acidosis in the absence (Control) and presence of afobazole when cells were exposed to acidosis in normal PSS (Control) or PSS containing 300 μM afobazole (Afob) or 300 μM afobazole + 10 nM BD 1063 (Afob + BD 1063). Asterisks denote significant difference from Control \(p<0.05\), and pound symbol indicates significant difference between the afobazole and the afobazole + BD 1063 groups \(p<0.05\). C, bar graph of percentage inhibition of peak (Peak) and net (Area) acidosis-evoked increases in [Ca\(^{2+}\)], produced when cells were treated with 300 μM afobazole (Afob) or with afobazole + 10 nM BD 1063 (Afob + BD 1063). Asterisks denote significant difference between Afob and Afob + BD 1063 groups for both peak and area \(p<0.001\). For all groups, \(n=125\).

(right traces), afobazole produced less of a block of the acidosis-evoked increases in [Ca\(^{2+}\)], relative to the cell incubated in PSS (left traces). Compiling several identical recordings demonstrated that preincubation in metaphor significantly decreased afobazole inhibition of both peak change in [Ca\(^{2+}\)], and the net [Ca\(^{2+}\)], increase observed in neurons in response to acidosis (Fig. 3B). Peak increase in [Ca\(^{2+}\)], was decreased by 40 ± 2% by 300 μM afobazole under control conditions (PSS preincubation) but by only 25 ± 1% after metaphor preincubation. Likewise, the inhibition of net increase in [Ca\(^{2+}\)], by afobazole was reduced from 41 ± 2% in control cells to 27 ± 1% when cells were preincubated in metaphor (Fig. 3C). These decreases were statistically significant \(p<0.001\).

To determine whether α-1 receptors were specifically involved in afobazole regulation of acid-evoked increases in [Ca\(^{2+}\)], the α-1-selective antagonist BD 1063 was used to disrupt afobazole signaling. Figure 4A shows representative traces of [Ca\(^{2+}\)], as a function of time recorded from a single cell during acidosis in the absence (Control) and presence of
300 μM afobazole (Afob) and 300 μM afobazole plus 10 nM BD 1063 (Afob + BD 1063). When afobazole was applied in the presence of BD 1063, the effects of afobazole on changes in [Ca$^{2+}$], were lessened. BD 1063 alone had no effects on acidosis-elicted increases in [Ca$^{2+}$], (Supplemental Fig. 1). In identical experiments, 10 nM BD 1063 decreased the effects of afobazole on acidosis-evoked changes in peak and net [Ca$^{2+}$], increases (Fig. 4B). Peak increases in [Ca$^{2+}$], were 160 ± 6 nM under control conditions but decreased to 71 ± 4 nM upon application of 300 μM afobazole. However, when afobazole was applied with BD 1063, the increase in [Ca$^{2+}$], was 105 ± 4 nM. Likewise, the control net increase in [Ca$^{2+}$], was reduced from 20 ± 1 to 8 ± 1 nM per min after afobazole application but increased to 15 ± 1 nM per min when BD 1063 was applied along with the afobazole. Thus, afobazole inhibition of peak increase in [Ca$^{2+}$], was reduced from 54 ± 2 to 30 ± 2%, and the block of net [Ca$^{2+}$], decreased from 55 ± 3 to 24 ± 6% in the presence of the σ-1 antagonist.

The ion channels, which stimulate acidosis-evoked increases in [Ca$^{2+}$], in cortical neurons, ASIC1a, also contribute to the elevations in [Ca$^{2+}$], in these neurons during ischemia (Mari et al., 2010). Therefore, experiments were carried out to ascertain whether afobazole could suppress ischemia-evoked [Ca$^{2+}$], overload. In response to in vitro ischemia, [Ca$^{2+}$], increased significantly and returned to baseline levels upon termination of the ischemic episode. In the same cell, when ischemic conditions were presented in the presence of afobazole, the elevation in [Ca$^{2+}$], was lower. Likewise, application of DTG decreased the elevations in [Ca$^{2+}$], observed in a cortical neuron after ischemia (Fig. 5A, bottom traces). However, higher concentrations of DTG were required to produce inhibitions equal to those by afobazole. Figure 5B shows concentration-response relationships for afobazole and DTG inhibition of ischemia-evoked [Ca$^{2+}$], overload. Afobazole reduced changes in [Ca$^{2+}$], after ischemia in a concentration-dependent manner. A fit of the data using a single-site, 3-parameter Langmuir-Hill equation indicates that afobazole is able to maximally inhibit [Ca$^{2+}$], overload by 69.4%. Half-maximal inhibition was observed at 1 μM afobazole and maximal inhibition at approximately 300 μM. In contrast, DTG inhibited the ischemia-induced [Ca$^{2+}$], overload with a half-maximal concentration of 75 μM. In the concentration range of 1 to 100 μM, afobazole produced a statistically greater block than DTG. However, DTG exhibited greater efficacy than afobazole by blocking over 70% of the response at the highest concentrations tested (Fig. 5B).

The role of σ receptors in the regulation of ischemia-evoked [Ca$^{2+}$], overload was assessed using metaphit. Figure 6A shows representative [Ca$^{2+}$], traces recorded in response to in vitro ischemia from two neurons. Whereas afobazole was found to decrease the ischemia-evoked [Ca$^{2+}$], overload under both conditions, the cell preincubated in metaphit responded to afobazole to a lesser extent. Similar experiments confirmed that preincubation in metaphit decreased the ability of afobazole to depress peak ischemia-evoked [Ca$^{2+}$], overload in cortical neurons (Fig. 6B). Afobazole depressed ischemia-evoked [Ca$^{2+}$], overload in control neurons by 68 ± 2 but only by 48 ± 4% after incubation in metaphit. This 29% reduction in the response to afobazole was statistically significant and consistent with a σ receptor-mediated effect.

To determine the specific σ receptor subtype involved in the modulation of ischemia-induced [Ca$^{2+}$], overload, experiments were carried out using the σ-1 receptor-selective antagonist BD 1047. A representative experiment on an isolated cortical neuron using this drug is shown in Fig. 7A. Application of 300 μM afobazole depressed the [Ca$^{2+}$], overload elicited by in vitro ischemia in the neuron. However, when afobazole was coapplied with 10 μM BD 1047, the effects of afobazole were reduced (Fig. 7A). Using this same protocol, it was determined that application of BD 1047 (10 μM) reduced the effects of afobazole on both peak and net elevations in [Ca$^{2+}$], in neurons (Fig. 7B). In the presence of BD 1047, the percentage inhibition of peak and net [Ca$^{2+}$], overload produced by 300 μM afobazole were reduced by 29 ± 4 and 34 ± 5%, respectively (Fig. 7C).

To date, the capacity of σ receptors to regulate [Ca$^{2+}$], overload elicited by in vitro ischemia in the neurons has only been studied for short term exposure to ischemic conditions.
Katnik et al., 2006; Mari et al., 2010), and thus, the effects of σ receptor activation of long-term [Ca^{2+}]_i homeostasis remains unknown. The ability of afobazole to preserve [Ca^{2+}]_i homeostasis was studied in neurons exposed to 1 h of ischemia. Figure 8 shows a bar graph of mean intracellular [Ca^{2+}]_i recorded from neurons incubated under normal and ischemic conditions in the absence and presence of afobazole. Application of afobazole for 1 h had no direct effects on intracellular [Ca^{2+}]_i. However, in neurons subjected to in vitro ischemia, afobazole significantly decreased mean [Ca^{2+}]_i. Thus, afobazole is able to decrease both the peak increases in [Ca^{2+}]_i, produced by short-term ischemia and mitigate the [Ca^{2+}]_i overload produced by extended ischemic events in neurons. Intracellular calcium overload is a key component to neuronal death caused by acidotoxicity and ischemia. Thus, the capacity of afobazole to mitigate [Ca^{2+}]_i, dysregulation under...
these conditions suggests that this drug may enhance neuronal survival in these pathophysiological states. Experiments were carried out to determine whether afobazole can decrease neuronal death in response to in vitro ischemia. Cultured neurons were exposed to a combination of oxygen-glucose deprivation and 4 mM azide for 2 h to stimulate ischemia-induced cell death. Neuronal death was quantified using an LDH cytotoxicity assay. Figure 9A shows the relative degree of cell death observed with or without ischemia under control conditions (no drug) and when DTG and afobazole were applied at the indicated concentrations (in micromolars). Both afobazole and DTG significantly decreased neuronal death after ischemia in a concentration-dependent manner. Consistent with data shown in Fig. 5, afobazole was more potent than DTG, whereby identical concentrations of afobazole decreased cell death to a greater extent than DTG. Further analysis of the data from Fig. 9A indicates that afobazole reduced neuronal death by 17 to 20% more than DTG at identical concentrations, with 100 μM afobazole decreasing neuronal death by 39 ± 1% (Fig. 9B).

Discussion

The primary discovery reported here is that afobazole acts on σ receptors to inhibit intracellular Ca2+ overload produced by acidosis, as well as short-term and long-term ischemia in cortical neurons. Moreover, the σ-1 receptor was shown to be specifically involved in the afobazole-mediated effects on acidosis-induced intracellular [Ca2+]i dysregulation. Afobazole was further shown to be effective at decreasing neuronal death after in vitro ischemia. Compared with the prototypical σ-receptor ligand DTG, afobazole was shown to be more potent at decreasing intracellular [Ca2+]i overload after ischemia. It is noteworthy that afobazole was shown to be a more potent neuroprotective agent than DTG in the cell survival assay.

Previous in vitro studies have shown that afobazole can decrease neuronal death in response to H2O2 and glutamate application (Zenina et al., 2005). However, afobazole had to
be introduced into the culture medium 30 min and 24 h before H2O2 and glutamate exposure, respectively, to provide neuroprotection. It was reported that administration of afobazole at concentrations as high as 10 μM after glutamate exposure failed to enhance neuronal survival. Previous data suggesting that higher concentrations of afobazole (10−7 M) provided less neuroprotection than lower concentrations (10−8 M) of the drug (Zenina et al., 2005), although statistical significance for this difference was not demonstrated. Thus, there is some confusion in the literature over the effectiveness of afobazole as a neuroprotective agent in neurons. Moreover, it remained unknown whether afobazole could decrease cell death in response to ischemia. Data presented here show that afobazole can effectively decrease cell death after ischemia in a concentration-dependent manner. Our data demonstrate that afobazole, at concentrations as high as 10−4 M, exerts neuroprotective effects in neurons. This concentration of afobazole (100 μM) decreased cell death by nearly 40%, which is an approximately 2-fold higher enhancement of survival than that previously reported for afobazole in the H2O2 and glutamate studies (Zenina et al., 2005).

The molecular mechanism(s) accounting for afobazole-mediated neuroprotection remained unknown. A recent study showed that afobazole decreased caspase-3 activity in response to glutamate excitotoxicity in the immortalized neuronal cell line HT-22 (Antipova et al., 2010). However, it is unclear whether afobazole is acting directly on caspase-3 or through some upstream signaling molecule. Another putative afobazole target for neuroprotection is σ receptors, which have been postulated as an effector for this drug (Seredenin et al., 2009). Afobazole has a pharmacophore structure similar to that of the σ-1-receptor agonist (+)-pentazocine. Binding studies showed that afobazole displaces (+)-pentazocine in Jurkat cells with an IC50 of 7 μM (Seredenin et al., 2009). Application of afobazole onto HT-22 cells results in translocation of σ-1 receptors from the cell body to axons, suggesting possible activation of the receptors by afobazole (Seredenin et al., 2009). However, before our study, it had not been clearly established whether afobazole is an agonist or antagonist of σ receptors. Our data show that inhibition of σ receptors with the irreversible antagonist metaphit prevents afobazole inhibition of both acidosis- and ischemia-evoked [Ca2+]i overload. The concentration of metaphit and the percentage inhibition of the afobazole effects produced are consistent with these effects mediated by σ receptors (Katnik et al., 2006; Herrera et al., 2008). Furthermore, it was found that the σ-1-receptor-selective antagonist BD 1063 inhibits the effects of afobazole on acidosis-evoked [Ca2+]i overload at a concentration indicative of a σ-1-receptor-mediated effect (Matsumoto et al., 1995; Herrera et al., 2008). Likewise, BD 1047, which is also selective for σ-1 (Matsumoto et al., 1995) and has been shown to disrupt σ-1-receptor-mediated inhibition of ischemia-induced [Ca2+]i overload (Katnik et al., 2006), blocked the effects of afobazole in our in vitro ischemia model. Taken together, our data suggest that afobazole acts as a σ-receptor agonist and that it specifically activates σ-1 receptors. Additional data collected in our laboratory suggests that afobazole may also act as a σ-2-receptor agonist (Cuevas et al., 2011). Given that neither metaphit nor the σ-1-receptor-selective antagonists completely eliminated the effects of afobazole, the possibility exists that this drug is also acting via mechanisms other than σ receptors to prevent [Ca2+]i overload. Such possible mechanisms remain to be explored.

By activating σ receptors, afobazole is likely to affect several channel types involved in acidotoxicity and ischemic injury. The [Ca2+]i overload occurring under these conditions is attributed to the activation of multiple plasma membrane ion channels, including acid-sensing ion channels, voltage-gated Ca2+ channels, and ionotropic glutamatergic receptors (Katnik et al., 2006; Herrera et al., 2008). Neuronal injury has been directly linked to some of these channels, such as the acid-sensing ion channel 1a (Xiong et al., 2004). σ Receptors couple to these various channel types and suppress their activity (Zhang and Cuevas, 2002, 2005; Katnik et al., 2006; Herrera et al., 2008; Zhang et al., 2009). Thus, the effects of afobazole are probably the result of a broad-spectrum inhibition of plasma membrane ion channels downstream of σ receptors. It is noteworthy that our data indicate that ASICs expressed in cortical neurons are inhibited by afobazole application. Both proton-evoked membrane currents and increases in [Ca2+]i were reduced by afobazole. These neurons functionally express homomeric ASIC1a and ASIC2a channels, as well as heteromeric express ASIC1aASIC2a channels (Askwith et al., 2004). The pH and PcTx1 sensitivity of the channels affected by afobazole suggests that the drug is probably acting on heteromeric ASIC1aASIC2a channels, because these channels are activated at pH 6.0 and are not inhibited by PcTx1 (Escoubas et al., 2003; Hesselager et al., 2004). ASIC1a-containing channels have been shown to be involved in anxiety and fear in various animal models (Wemmie et al., 2004; Coryell et al., 2007). Thus, afobazole modulation of ASIC probably contributes to the anxiolytic properties of this drug.

Several studies have reported that afobazole is neuroprotective in vivo. In rats undergoing phototriobiosis of vessels in the prefrontal cortex, application of afobazole 1 h after the onset of ischemic stroke and for 8 days thereafter reduced stroke volume by ~50% (Seredenin et al., 2008). Likewise, in two hemorrhagic stroke models, afobazole increased survival and improved motor outcomes when applied 3 to 6 h after onset of stroke and then twice daily for 2 weeks (Galaeva et al., 2005; Kraineva and Seredenin, 2010). The in vitro observations presented here suggest that afobazole activation of σ receptors in vivo may contribute to the neuroprotective properties of this compound in both ischemic and hemorrhagic stroke. Our laboratory has previously shown that activation of σ receptors with DTG can significantly decrease stroke volume in the middle cerebral artery occlusion rat model of ischemic stroke (Ajmo et al., 2006). Data shown here indicate that afobazole is a more potent neuroprotectant than DTG in an in vitro ischemia model. It remains to be determined whether afobazole is more potent than DTG in vivo. Important from a therapeutic perspective, activation of σ receptors provides neuroprotection at delayed time points (Ajmo et al., 2006). It is of significant interest to determine whether afobazole can also decrease brain injury after stroke at delayed time points and, thus, whether it can extend the therapeutic window for effective stroke treatment.

In conclusion, afobazole effectively reduces [Ca2+]i overload elicited by acidosis and in vitro ischemia in rat cortical neurons. Afobazole acts as an agonist at σ receptors to block these [Ca2+]i elevations. The inhibition of [Ca2+]i overload
during acidosis by afobazole was specifically linked to activation of σ-1 receptors by the compound. Afobazole was shown to be neuroprotective during ischemia, and afobazole-mediated inhibition of $[Ca^{2+}]_i$ overload probably accounts, at least in part, for the neuroprotection reported here. Given that afobazole has been shown to be safe when used clinically for anxiety, this drug represents a viable candidate for stroke therapy. Moreover, the effectiveness of σ-receptor activation for neuroprotection at delayed time points after stroke suggests that afobazole may expand the therapeutic window for effective stroke therapy in humans.

Acknowledgments

We thank Drs. Andrei Petrov and Ilia Yasnys and Nivia Cuevas for comments on a draft of this article.

Authorship Contributions

Participated in research design: Cuevas and Katnik.

Conducted experiments: Cuevas, Behensky, Deng, and Katnik.

Performed data analysis: Cuevas, Behensky, Deng, and Katnik.

Wrote or contributed to the writing of the manuscript: Cuevas, Behensky, and Katnik.

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