Methazolamide and Melatonin Inhibit Mitochondrial Cytochrome C Release and Are Neuroprotective in Experimental Models of Ischemic Injury

Xin Wang, PhD; Bryan E. Figueroa, MD; Irina G. Stavrovskaya, PhD; Yi Zhang, PhD; Ana C. Sirianni, MS; Shan Zhu, PhD; Arthur L. Day, MD; Bruce S. Kristal, PhD; Robert M. Friedlander, MD, MA

Background and Purpose—The identification of a neuroprotective drug for stroke remains elusive. Given that mitochondria play a key role both in maintaining cellular energetic homeostasis and in triggering the activation of cell death pathways, we evaluated the efficacy of newly identified inhibitors of cytochrome c release in hypoxia/ischemia induced cell death. We demonstrate that methazolamide and melatonin are protective in cellular and in vivo models of neuronal hypoxia.

Methods—The effects of methazolamide and melatonin were tested in oxygen/glucose deprivation–induced death of primary cerebrocortical neurons. Mitochondrial membrane potential, release of apoptogenic mitochondrial factors, pro–IL-1β processing, and activation of caspase -1 and -3 were evaluated. Methazolamide and melatonin were also studied in a middle cerebral artery occlusion mouse model. Infarct volume, neurological function, and biochemical events were examined in the absence or presence of the 2 drugs.

Results—Methazolamide and melatonin inhibit oxygen/glucose deprivation–induced cell death, loss of mitochondrial membrane potential, release of mitochondrial factors, pro–IL-1β processing, and activation of caspase-1 and -3 in primary cerebrocortical neurons. Furthermore, they decrease infarct size and improve neurological scores after middle cerebral artery occlusion in mice.

Conclusions—We demonstrate that methazolamide and melatonin are neuroprotective against cerebral ischemia and provide evidence of the effectiveness of a mitochondrial-based drug screen in identifying neuroprotective drugs. Given the proven human safety of melatonin and methazolamide, and their ability to cross the blood-brain-barrier, these drugs are attractive as potential novel therapies for ischemic injury. (Stroke. 2009;40:1877-1885.)

Key Words: methazolamide ■ melatonin ■ neuroprotection ■ ischemic stroke

Release of cytochrome c from the mitochondria triggers a set of events leading to the activation of programmed cell death.1,2 Cytochrome c release occurs in a variety of neurological diseases, including stroke, Huntington disease, and amyotrophic lateral sclerosis.3–5 Inhibition of cytochrome c is therefore a potential therapeutic target for neuroprotection. To identify novel neuroprotective agents, we screened a library of FDA-approved drugs for inhibition of cytochrome c release. Cytochrome c release can be induced in purified mitochondria, providing a screening method for the identification of compounds which could inhibit this process.

We identified a number of drugs that inhibit cytochrome c release in purified mitochondria as well as in mutant Huntington-expressing striatal cells.6 One of these drugs, methazolamide, is neuroprotective in vivo in a transgenic mouse model of Huntington disease.6 To determine whether this screen identified protective drugs for acute neurodegenerative models, we tested 2 drugs in an experimental model of cerebral ischemia. Here we provide evidence for the neuroprotective properties of 2 of the drugs identified in the screen, methazolamide and melatonin, both in primary cerebrocortical neurons (PCNs) exposed to several cell death stimuli, as well as in vivo in cerebral ischemia. Neuroprotection in vivo is associated with inhibition of cytochrome c release and of caspase-3 activation. This report provides evidence of the efficacy of a mitochondrial-based screen in identifying drugs that are protective in an acute model of neurodegeneration and provides further support for the importance of cytochrome c release in the pathogenesis of ischemic injury.
Materials and Methods

Drugs
Methazolamide and melatonin were obtained from Sigma.

Cell Lines and Induction of Cell Death
Culture of PCNs were isolated as previously described and subjected to oxygen-glucose deprivation (OGD), 1 mmol/L H2O2, or 500 μmol/L NMDA for 18 hours. All cultures were used between days 7 to 10 after harvest. Cell death was determined by lactate dehydrogenase assay. Morphology of PCN cells was observed, chromatin condensation and nuclear fragmentation were analyzed with Hoechst 33342 staining (Molecular Probes; 1:5000 for 2 to 5 minutes).

Lactate Dehydrogenase Assay
The assay was performed as previously described according to the manufacturer’s instructions (Roche).4

Terminal dUTP Nick-End Labeling Assay
The assay was performed using the DeadEnd Fluorometric terminal dUTP nick-end labeling (TUNEL) system (Promega) as specified by the manufacturer. Briefly, attached PCNs were fixed with 4% methanol-free formaldehyde, permeabilized by 0.2% Triton-X-100, and incubated with the TUNEL reaction mixture for 1 hour at 37°C. After thorough washes, chromatin condensation and nuclear fragmentation were analyzed using a Nikon ECLIPSE TE-200 fluorescence microscope.

Western Blot
Mouse brain samples were lysed in RIPA buffer with protease inhibitors. Antibody to caspase-3 was purchased from Cell Signaling Technology, antibody to caspase-1 from Santa Cruz Biotechnology, and antibody to β-actin from Sigma.

Cellular and Tissue Fractionation
PCNs and mouse brain cytosolic fractions were performed as described.7 Released cytochrome c or AIF was analyzed by Western blot. Antibody to cytochrome c was purchased from PharMingen, and to AIF from Sigma.

Mature IL-1β Determination
Mature IL-1β quantification was performed as previously described4 by using an ELISA kit specific for the mature form of the cytokine (R&D Systems).

Caspase-1 Activity Assay
Cell extracts and enzyme assays were performed with the ApoAlert caspase fluorescent assay kit as previously described. Caspase-1-like substrate Ac-YVAD-AFC (7-amino-4-trifluoromethylcoumarin) was purchased from Calbiochem. Released AFC was quantified in a Bio-Rad Versa Fluorometer (excitation at 400 nm and emission at 505 nm).

Determination of Mitochondrial Transmembrane Potential (ΔΨm)
PCNs were treated as indicated with or without methazolamide, or melatonin. Living cells were stained with Rhodamine 123 (Rh 123, Molecular Probes) as previously described.5,6

Measurement of Mitochondrial Permeability Transition
Rat liver mitochondria were isolated and treated as previously described.6,9

Permanent Middle Cerebral Artery Occlusion
Focal cerebral ischemia was produced as previously described.4,7 Briefly, anesthesia was induced in 6- to 8-week-old C57BL/6J mice (body weight 20 to 24 g, Jackson Laboratory, Bar Harbor, Maine) with 2% (vol/vol) isoflurane (70% N2O/30% O2) and was maintained with a 1 to 0.5% concentration. Rectal temperature was maintained between 37.0 and 37.5°C with a heating pad (Harvard Apparatus). Focal cerebral ischemia was induced by an intraluminal 7.0-mm thread with a silicone tip (180 μm diameter) introduced into the right cerebral internal carotid artery. The thread was inserted 9±1.0 mm into the internal carotid artery up to the middle cerebral artery. A laser Doppler perfusion monitor (Perimed AB) was adhered to the right temporal aspect of the animal’s skull and used to confirm middle cerebral artery flow disruption. Control animals underwent sham surgery consisting of only anesthesia and carotid artery dissection. Each animal was euthanized after surgery. The brain was removed, and the cerebral hemisphere was retained for either evaluation of infract volume or Western blot analysis.

Drug Treatment in Middle Cerebral Artery Occlusion Model
Control mice were treated with a normal saline vehicle control containing 0.4% DMSO and 3% Tween-20. Methazolamide and melatonin were respectively prepared in normal saline containing 0.4% DMSO or 3% Tween-20 and administered by intraperitoneal injection at a dose of 20 mg/kg for methazolamide and 10 mg/kg for melatonin. Mice were treated either 1 hour before 30 minutes after permanent middle cerebral artery occlusion (MCAO).

Determination of Infarct Volume
After 24 hours of MCAO, the mice were euthanized. Brains were rapidly removed and chilled for 2 minutes. Coronal sections (1-mm thick; n=7) were cut with a mouse brain matrix. Each slice was immersed in saline solution containing 2% 2,3,5-triphenyltetrazolium chloride (Sigma) at 37°C for 20 minutes. After staining, each slice was scanned with an HP scanner 4200C. The stained and unstained areas of the right hemisphere were quantified with imageJ 1.32j (NIH), and these values were used to calculate the infarct volume expressed as a percentage of the lesional hemisphere.

Neurobehavioral Examination
Mice were assigned a neurobehavioral score at 24 hours after MCAO. A previously reported scale was used.7,10

Statistical Analysis
Densitometric quantification was performed with the Quantity One Program (Bio-Rad). Statistical significance was evaluated by t test: *probability values <0.05 were considered significant; **probability values <0.001. Drug analysis, including IC50, and maximum protection, were performed using the GraphPad Prism program.

Results
Methazolamide and Melatonin Are Neuroprotective in Oxygen Glucose Deprivation–Mediated Primary Cerebrocortical Neuronal Death
Using a purified mitochondrial screen, we identified drugs that inhibit release of cytochrome c.6 To determine whether this screen is effective at identifying drugs that are neuroprotective in acute cell death, we evaluated two of the “hits,” methazolamide and melatonin, in an in vitro model of cerebral ischemia. Exposing PCNs to OGD induces cytochrome c release and caspase-3 activation, ultimately leading to cell death.5,11 Incubation with either methazolamide (100 nmol/L to 100 μmol/L) or melatonin (1 μmol/L to 100 μmol/L) resulted in statistically significant inhibition of OGD-mediated PCN cell death (Figure 1A and 1C). To determine the relative potencies of the 2 drugs, we measured the extent of cell death as a function of drug concentration. The resulting curves...
Methazolamide and Melatonin Protect PCNs From a Variety of Cell Death Inducers

As described above, methazolamide and melatonin inhibit OGD-induced PCN cell death. We next evaluated whether methazolamide- or melatonin-mediated neuroprotection could be extended to additional cell death paradigms. H2O2- and NMDA-induced cell death have been used as in vitro models of oxidative damage and excitotoxic neuronal injury.7,8,12,13 We therefore evaluated the ability of methazolamide and melatonin to protect PCNs challenged with H2O2 or NMDA. Methazolamide and melatonin inhibited both H2O2- and NMDA-induced PCN cell death in a dose dependent manner (Figure 2A, 2B, 2D, and 2E). Phase-contrast photomicrographs demonstrate H2O2-mediated loss of PCN neuritic processes (Figure 2G, lower panel), cell death-associated nuclear fragmentation, and chromatin condensation (arrows in Figure 2G, upper panel). Methazolamide (Figure 2A and 2B) and melatonin (Figure 2D and 2E) significantly inhibited cell death induced by either stimulus. Phase-contrast micrograph demonstrates that both methazolamide and melatonin not only inhibited cell death, but also partially restored normal cellular morphology. Note that Figure 2 also includes data on the cytochrome c release inhibitor minocycline (Figure 2C and 2F). Minocycline is used here as a positive control, given its demonstrated neuroprotective properties as an inhibitor of cytochrome c release.8,14,15 To provide further evidence of modulation of cell death, we performed TUNEL labeling and evaluated the neurons using a fluorescence microscope (Figure 2H).

TUNEL staining revealed a clear increase of chromatin condensation and fragmentation in H2O2-treated apoptotic nuclei of PCNs as compared to control cells. Incubation of methazolamide and melatonin reduced the degree of TUNEL-positive cells. Having observed that methazolamide and melatonin prolong the survival of PCNs subjected to OGD, H2O2, and NMDA, we proceeded to study what cell death–associated molecular changes they affect.

Inhibition of Mitochondrial Cell Death Pathways Contributes to Neuroprotection by Methazolamide and Melatonin

Our observation that methazolamide is neuroprotective in PCNs is the first in the literature. Now it becomes of interest to determine the effects of the compound on the molecular physiology of the cell. The ability of melatonin to inhibit neuronal cell death has been previously demonstrated,16–21 but the signaling mechanisms mediating the neuroprotective actions of melatonin remain poorly understood. Given that methazolamide and melatonin were identified by their ability to inhibit cytochrome c release from purified mitochondria, we next evaluated whether these drugs could inhibit OGD-mediated cytochrome c release and caspase-3 activation.

The release of cytochrome c and apoptosis-inducing factor (AIF) from mitochondria trigger downstream caspase-3 activation and additional caspase-independent cell death events.1,22 Blocking the release of apoptogenic factors into the cytoplasm should inhibit cell death. Indeed, as demonstrated by Western blotting, OGD induces mitochondrial release of cytochrome c and AIF (Figure 3A and 3B). Methazolamide (Figure 3A) and melatonin (Figure 3B) effectively inhibited release of these cell death mediators. We next evaluated the activity of the downstream effector caspase-3. Western blot analysis demonstrated that
Figure 2. Methazolamide and melatonin inhibit cell death of primary cerebrocortical neurons under exposure to H$_2$O$_2$ or NMDA. Cell death of PCNs was induced by 18-hour exposure to 1 mmol/L H$_2$O$_2$ (A through C and G) or 500 µmol/L NMDA (D through F and G). A through F. Cell death was evaluated by the lactate dehydrogenase assay. Data from 3 independent experiments are graphed, and statistically significant differences are indicated with * if $P<0.05$ and ** if $P<0.001$. White, black, and gray bars are labeled as indicated in Figure 1. G. Phase-contrast light micrographs of control versus H$_2$O$_2$-treated PCNs with or without incubation with methazolamide (10 µmol/L) or melatonin (10 µmol/L) are shown as indicated (middle panel). Nuclei are stained with Hoechst 33342 (upper panel).
Caspase-3 was activated on OGD induction, and both methazolamide and melatonin effectively inhibited caspase-3 activation (Figure 3C).

**Methazolamide and Melatonin Slow Dissipation of the Mitochondrial Membrane Potential Gradient in Primary Cerebrocortical Neurons**

Proper mitochondrial membrane potential ($\Delta \psi_m$) is critical for appropriate cellular bioenergetic homeostasis, and its loss is an important event associated with progression of mitochondrial dysfunction and leading to cellular demise. We therefore evaluated whether, given their neuroprotective properties, methazolamide and melatonin could inhibit the dissipation of $\Delta \psi_m$. Comparing with the punctate Rh123 staining seen in normal $\Delta \psi_m$ in control PCNs, OGD results in a diffuse and lower intensity staining pattern as previously described (Figure 4A, upper panel). Both methazolamide and melatonin inhibited OGD-associated loss of mitochondrial $\Delta \psi_m$ (Figure 4A, lower panel). Hence methazolamide and melatonin treated PCNs exposed to OGD maintain proper $\Delta \psi_m$, therefore pointing to a mechanism of action involving not only inhibition of release of apoptogenic factors, but also preservation of appropriate cellular energetics.

**Melatonin Is Not a Mitochondrial Permeability Transition Inhibitor in Isolated Mitochondria**

The mitochondrial permeability transition pore consists of a multimeric complex of proteins spanning the inner and outer membranes. Its opening is a component of mitochondrial dysfunction, itself a result of such biochemical stresses as high concentrations of Ca$^{2+}$ ion, oxidizing agents, thiol reactive compounds (ie, reactive aldehyde), and proapoptotic cytosolic proteins. The loss of $\Delta \psi_m$ is an early event in the cell death pathway and an important trigger of the caspase cascade.

There is no satisfactory method for directly determining the mitochondrial permeability transition in living cells. Consequently, we investigated the effects of methazolamide on this molecular event in purified mitochondria. In these experiments, mitochondrial permeability transition (mPT) was induced by Ca$^{2+}$ ions, the oxidizing agent organic hydroperoxide tBH, the thiol cross-linking agent PhAsO, the thiol oxidant diamide, or a combination thereof (Figure 4B, and 4C). Melatonin, similar to methazolamide, did not protect purified mitochondria from these chemical challenges. These observations contrast with the analogous ones using (1) minocycline, a compound that blocks the loss of the mitochondrial membrane potential both in vitro and in vivo, or (2) the heterocyclic, tricyclic, or phenothiazine-derived agents.

Neither inhibition of the mPT nor direct effects on mitochondrial physiology appear to underlie the protection imparted by melatonin. Initial studies in isolated liver mitochondrial focused on monitoring swelling as a marker or mPT induction and tested the agents at a broad dose response range. No effects were seen, suggesting that these agents do not interfere with mPT induction across a broad concentration range. Because work in other systems, such as cultured cerebellar granular neurons, had suggested that interference with mitochondrial physiology through uncoupling and respiratory inhibition could also appear protective, we examined the effects of melatonin on basic physiological properties. Studies were done on a recently adapted fluorescence system that allows simultaneous measurement of membrane potential (by following TMRM fluorescence), Ca$^{2+}$ ions, the oxidizing agent organic hydroperoxide tBH, the thiol cross-linking agent PhAsO, the thiol oxidant diamide, or a combination thereof (Figure 4B, and 4C). Melatonin, similar to methazolamide, did not protect purified mitochondria from these chemical challenges. These observations contrast with the analogous ones using (1) minocycline, a compound that blocks the loss of the mitochondrial membrane potential both in vitro and in vivo, or (2) the heterocyclic, tricyclic, or phenothiazine-derived agents.

Melatonin did not have any significant effects on $\Delta \psi_m$, calcium transport, NAD$^+$/NADH redox status, or swelling (Figure 4B). Thus, the data in Figure 3B and 3C suggest that melatonin does not mediate its protective effect by acting on basic mitochondrial physiology, including mitochondrial redox status and substrate, proton, electron, or Ca$^{2+}$ transport. These data are most consistent with effects mediated outside the inner mitochondrial membrane/mitochondrial matrix, including potential targets such as bcl-2 family members (eg, bakh/bax) and the elements involved in release of proapoptotic proteins. Studies are in progress to address these potential mechanisms.

**Figure 2.** (Continued) Arrows point to apoptotic cells with condensed or fragmented chromatin. Bar=5 $\mu$m. H, TUNEL staining in cells treated with H$_2$O$_2$ reveals a large increase of positive nuclei with condensed chromatin and DNA fragmentation as compared to control cells. Incubation with methazolamide (10 $\mu$mol/L) or melatonin (10 $\mu$mol/L) significantly reduces the numbers of TUNEL-positive cells. Scale Bar=5 $\mu$m.
Melatonin and Methazolamide Inhibit OGD-Induced Mature IL-1β Release and Caspase-1 Activation

Caspase-1 plays a critical role as an apical activator of OGD-induced PCN death. The release of mature IL-1β serves as an indicator of caspase-1 activation. Endogenously produced IL-1β plays a role in a variety of cell death paradigms, and the inhibition of cleavage of pro-IL-1β and the secretion of mature IL-1β are associated with inhibition of cell death. To further evaluate the mechanisms of
action of methazolamide and melatonin, using an ELISA assay specific for the mature form of IL-1β, we quantified the release of the active cytokine into conditioned medium of OGD-treated PCNs. Consistent with previous evidence that OGD treatment induces caspase-1 activation and IL-1β processing in PCNs, we detected a greater than 4-fold increase in the concentration of mature IL-1β release after the completion of OGD (A and B), or whole cells were extracted (C and D), and analyzed by Western blot (each of which contained 50 μg of protein) using antibodies to caspase-1 (C). Beta-actin was used as a loading control. This blot is representative of 3 independent experiments. Caspase-1 activity was quantified using a fluorogenic assay in lysed cells. Results are the average of at least 3 independent experiments. *P<0.05, **P<0.001.

**Figure 5.** Methazolamide and melatonin inhibit the release of IL-1β and caspase-1 activation. Cell death was induced in PCNs by subjecting cells to OGD for 3 hours with or without 10 μmol/L methazolamide (A, C, and D) and 10 μmol/L melatonin (B, C, and D). Subsequently, conditioned media was collected and assayed for mature IL-1β release after the completion of OGD (A and B), or whole cells were extracted (C and D), and analyzed by Western blot (each of which contained 50 μg of protein) using antibodies to caspase-1 (C). Beta-actin was used as a loading control. This blot is representative of 3 independent experiments. Caspase-1 activity was quantified using a fluorogenic assay in lysed cells. Results are the average of at least 3 independent experiments. *P<0.05, **P<0.001.

**Discussion**

Consequences of cerebral ischemia result in significant human morbidity and mortality. Despite great efforts to identify protective drugs for cerebral ischemia, discovery of an effective agent remains elusive. Failure might be in part the result of selection of inappropriate targets or the inappropriate executions and expectations of clinical trials. Given the prominent role of mitochondria both in generating energy required for cell survival and in triggering cell death pathways, we hypothesize that identifying drugs that target mitochondrial pathways may be broadly neuroprotective. Using an isolated/cell-free mitochondrial screen, we have identified a set of drugs that inhibit cytochrome c release in the isolated organelle. We demonstrate that most but not all the cytochrome c release inhibitors are protective in a number of neuronal cell death models. The 2 drugs selected for further evaluation, methazolamide and melatonin, not only inhibited cytochrome c release and cell death, but also inhibited the loss of mitochondrial membrane potential, caspase-3 activation, caspase-1 activation, and IL-1β processing associated with exposure to a cell death stimulus.

**Drug-mediated preservation of membrane potential under circumstances that otherwise would lead to cellular demise point to the ability of these agents to protect/enhance mitochondrial function. Therefore the drugs not only inhibit the release of mitochondrial cytochrome c/AIF with the subsequent activation of cell death pathways, but concomitantly**
improve mitochondrial function and as a result cellular health. Given that AIF is an important mediator of caspase-independent cell death pathways, our observation with methazolamide and melatonin inhibiting AIF release represents the first report demonstrating the ability to inhibit both caspase-dependent (cytochrome c) and caspase-independent (AIF) mitochondrial cell death pathways.

There are at least 2 major pathways for cytochrome c release in both in vitro and in vivo. These are the mPT and the proapoptotic pathways induced by Bcl-2 family members such as Bid/Bax/Bak/bad, etc. Given that these drugs do not act at the mPT, it is most likely that they act by a bcl-2 family–modulated pathway. Further refinement of the molecular target is a goal of ongoing and future research.

We demonstrate that these 2 drugs are protective in an in vivo experimental model of cerebral ischemia. This is the first demonstration that the carbonic anhydrase inhibitor methazolamide is neuroprotective in cerebral ischemia. The exogenous administration of methazolamide in the experimental stroke model reduced infarct volume, improved the neurological score, and lowered cytochrome c release and caspase-3 activation. Even though melatonin has been previously universally demonstrated to be neuroprotective in experimental models of cerebral ischemia in several species including mice,17 adult18 and neonatal rats,19 gerbils,20 and cats,21 the mechanisms of action are not known understood. Hence we extend this finding by demonstrating not only efficacy in a mouse model of cerebral ischemia but also inhibition of cytochrome c release as a target of melatonin-mediated neuroprotection. An additional new finding is the demonstration that melatonin inhibits hypoxia-induced caspase-1 activation. From a mechanistic and drug development standpoint, we demonstrate that a mitochondrial-based drug screen can identify effective neuroprotective drugs. Therefore, this work provides evidence of the efficacy of a mitochondrial screen in identifying drugs with neuroprotective properties. Given that the drugs were identified as inhibitors of cytochrome c release, this work also provides further support for the key role of this biological event in neurodegeneration. At the physiological level, it validates targeting the molecular process as a rational approach to the design of therapies for cerebral ischemia. We demonstrate that the mitochondrial screen is efficacious at selecting protective drugs not only for acute but also for chronic neurodegeneration. It is important to note that these drugs are safe, have been in human use for many years, and cross the blood–brain barrier, making them attractive agents for further human evaluation.

Sources of Funding

This work was supported by grants from the National Institutes of Health/National Institute of Neurological Disorders and Stroke (to
Disclosures

None.

References

Methazolamide and Melatonin Inhibit Mitochondrial Cytochrome C Release and Are Neuroprotective in Experimental Models of Ischemic Injury

Stroke. 2009;40:1877-1885; originally published online March 19, 2009; doi: 10.1161/STROKEAHA.108.540765

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/40/5/1877

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/