

Aequorin Protects Adult and Aging Hippocampal CA1 Neurons From Ischemic Cell Death

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ABSTRACT

During ischemia, excessive calcium influx through glutamate receptors can rapidly trigger cell death. Calcium binding proteins (CaBPs) may serve an important role in buffering neurons from potentially toxic elevations in the concentration of intracellular calcium. Previous work has demonstrated that hippocampal neurons expressing the CaBP calbindin-D28k are better able to withstand an excitotoxic insult than neurons lacking calbindin. We have been investigating the feasibility of regulating calcium levels in ischemia by replenishing CaBPs. Aequorin (AQ) is a 22 kDa CaBP isolated from the coelenterate *Aequorea victoria*. Our previous work (Detert et al., 2006) suggested that intrahippocampal infusion of AQ protects adult neurons from an ischemic insult. Studies have demonstrated a decrease in neuronal CaBPs (e.g., calbindin) with aging, so it is possible that older animals may be differentially affected by ischemia and AQ treatment. To evaluate potential age-related differences in the efficacy of AQ pretreatment, the present studies were done using four age groups. Adult (m = 5.3 mo), early middle-aged (m = 12.3 mo), late middle-aged (m = 20.0 mo) and aged (m = 24.8 mo) rats were stereotaxically implanted with bilateral cannula (in CA1 of the dorsal hippocampus) under aseptic conditions. After recovery, rats received an intrahippocampal infusion of 0.4% AQ or vehicle (0.5 μ l/min for 1 min). Twenty-four hours following the infusion, coronal brain slices (400 μ m) were cut with a vibratome. Slices were maintained in oxygenated artificial CSF (aCSF) for 1 hr. They were then subjected to a 5-min oxygen-glucose deprivation (OGD), returned to oxygenated-aCSF (with 0.2% trypan blue) for a 30-min reperfusion and then rinsed in oxygenated aCSF. All slice experiments were carried out at 35 °C. Slices were then fixed, sub-sectioned (40 μ m), mounted, and coverslipped. The number of trypan blue stained CA1 neurons was counted by an individual blind to both age and treatment group. Analysis of the data indicated that AQ treatment prior to OGD resulted in significantly fewer trypan blue stained CA1 neurons relative to control (AQ: 56 ± 6 ; control: 78 ± 6). A two-way ANOVA revealed a statistically significant effect of treatment [$F(1,120) = 6.5, p < .05$], however, there was no significant effect of age [$F(3,120) = 0.22, p = .88$] nor was there a significant age by treatment interaction [$F(3,120) = 1.65, p = .18$]. These data support the hypothesis that AQ may be an effective neurotherapeutic against ischemia when administered within 24 hours prior to an ischemic insult. It remains to be determined whether delivery of AQ is neuroprotective when administered *following* an ischemic insult.

INTRODUCTION

Calcium (Ca²⁺) plays a pivotal role in various neuronal processes, including neurotransmitter release (Lin & Scheller, 2000), gene expression, and synaptic plasticity (West et al., 2001). Neurons are continuously subjected to elevations in intracellular Ca²⁺ as a result of ongoing activity and this elevation is necessary for certain normal neuronal processes to occur, however too much Ca²⁺ can be toxic (Bano et al., 2005; Choi, 1992; Lee et al., 1999). Thus, the intracellular Ca²⁺ concentration in neurons is very tightly regulated (Kristian & Siesjo, 1998). Several mechanisms enable neurons to limit or control cytosolic Ca²⁺ (Baimbridge et al., 1992; Chard et al., 1993). In particular, calcium binding proteins (CaBPs) are important for binding and buffering cytosolic Ca²⁺.

Studies in the hippocampus (HPC) have shown that the presence of CaBPs confers some protection against excitotoxic insults, which would normally kill the cell (Gary et al., 2000). Furthermore, decreased levels of CaBPs are observed with advancing age (De Jong et al., 1996;

Krzywkowski et al., 1996; Villa et al., 1994), and in neurodegenerative disorders (Mattson & Magnus, 2006), including Alzheimer's disease (Hof & Morrison, 1991; Iacopino & Christakos, 1990; Mikkonen et al., 1999; Sutherland et al., 1993), Parkinson's disease (Iacopino & Christakos, 1990), and ischemia (Yenari et al., 2001).

During ischemia, neurons are subjected to excess Ca²⁺ influx triggering a cascade of events leading to cell death (Choi, 1992). A large rise in intracellular Ca²⁺ leads to necrosis, while moderate increases can lead to apoptosis (Ankarcrona et al., 1995). The cell death mechanism following ischemia is commonly thought to be necrosis first followed by apoptosis, possibly due to the differences in Ca²⁺ concentrations at different time points (Ueda & Fujita, 2004; Zipfel et al., 2000). Since neuronal CaBPs are depleted in neurodegenerative disorders, and since neurons that express CaBPs are better able to survive an excitotoxic challenge, we reasoned that supplementing with CaBPs prior to an ischemic insult might be neuroprotective. Furthermore, data from rodent studies suggest that aging-related decreases in CaBPs begin during middle age (Villa et al., 1994; Moyer et al., 2001), when animals are only beginning to show learning and memory deficits (Moyer & Brown, 2006). One possible explanation is that the decrease in neuronal CaBPs that occurs in middle-aged animals may ultimately leave those neurons more vulnerable to neurodegeneration in old age.

Treatments aimed at minimizing Ca²⁺ toxicity during ischemia have been administered before or after an ischemic insult, with positive results. For example, Yenari et al. (2001) treated animals with calbindin prior to inducing ischemia and found that over expression of calbindin resulted in fewer dead neurons. Also, Fan et al. (2007) treated rats with calbindin prior to ischemia and found a smaller infarct volume, better behavioral recovery, and decreased apoptosis in the calbindin-treated animals.

Aequorin (AQ) is a CaBP isolated from the coelenterate *Aequorea Victoria*. AQ has been used for years as an indicator of Ca²⁺ levels and has been shown to be safe and well tolerated by cells (Cobbold & Lee, 1991). AQ belongs to the EF-hand family of CaBPs, like calbindin, with EF-hand loops that are closely related to CaBPs in mammals (Toma et al., 2005). However, to date, no studies have investigated the therapeutic potential of AQ. Since HPC neurons are vulnerable to cell death following ischemia (Kirino & Sano, 1984), we tested the hypothesis that supplementing CaBPs in the HPC will be neuroprotective when administered prior to an ischemic insult. The present studies were designed to test this hypothesis by injecting AQ directly into the HPC, inducing ischemia, and comparing the number of dead cells between AQ- and controlinjected animals.

METHODS

Animals. Twenty-eight male F344 rats were used. The rats were from 3 different age groups: adult (3-7 mo), middle-aged (9-21 mo), and aged (22-28 mo).

Surgery. Rats were anesthetized with isoflurane and mounted on a stereotaxic apparatus. Under aseptic conditions, bilateral stainless steel guide cannulae were implanted in the dorsal hippocampus (relative to bregma: AP -3.5 mm, L ± 2.6 mm, V -3.0 mm). Cannulae were secured to the skull with stainless steel screws and epoxy. A stainless steel cap remained in place to prevent the occlusion of the guide cannulae.

Drugs and Infusions. Rats were given an infusion of either 0.02% AQ (w/v; Sigma-Aldrich) or aCSF 18-24 hours prior to decapitation. To facilitate neuronal uptake of AQ, 6% DMSO was included. Rats received bilateral infusions (0.5 µl/side) over 60 s and the injection cannulae remained in place for an additional 2 min. One hemisphere received an AQ infusion and the other an aCSF infusion. The infusion cannulae were cut to extend 0.5 mm beyond the guide cannulae.

Slice Preparation. 400 µm thick slices were prepared using standard procedures (Moyer & Brown, 1998). Following recovery, slices were subjected to a 5-min oxygen glucose deprivation (OGD) to induce ischemia. Briefly, slices were transferred to fructose CSF (fructose substituted for glucose)

that was bubbled with 95% N₂ / 5% CO₂. Following OGD, slices were then placed into an oxygenated aCSF solution containing 0.2% trypan blue for 30 minutes. Trypan blue readily penetrates dead and dying cells and stains them blue while leaving living cells unstained (DeRenzi & Schechtman, 1973). The slices were then briefly rinsed in oxygenated aCSF and fixed in 10% neutral buffered formalin overnight in the refrigerator. The next day, slices were cryoprotected, cut on a cryostat (40 µm), and mounted onto gelatin-coated slides.

Cell Counts. The slices were examined under an Olympus microscope (equipped with a digital camera) at 10X, and pictures were taken. Trypan blue stained neurons within CA1 (about an 800 µm section) were counted by an individual blind to treatment condition. An ANOVA was used to evaluate a drug and age effects (Statview v 5.0; SAS Institute, Inc., Cary, NC).

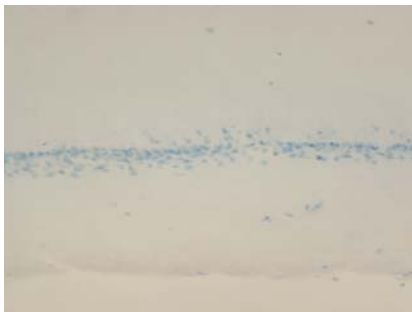
Active Caspase-3 Immunofluorescence. Following ischemia reperfusion in aCSF, slices were fixed in 4% paraformaldehyde, cryoprotected, and subsectioned (50 µm) on a cryostat. Slices were incubated in 1% NaBH₄ for 15 minutes and washed with PBS for 10 minutes (two times). Slices were incubated in 10% NGS, 5% BSA, and 0.2% Triton-X 100 for 30 minutes followed by primary antibody solution (1:100 rabbit anti-active caspase-3 [Biovision]) overnight at room temperature. The next day, slices were washed in PBS (10 min, twice) and incubated for 2 hr in secondary antibody solution in the dark (10 µg/mL anti-rabbit Ig-Alexa 594 [Molecular Probes]). The slices were then washed with PBS for 5 min (two times), rinsed in dH₂O, incubated for 10 min in CuSO₄, rinsed with dH₂O, and rinsed in PBS (5 min, twice). They were mounted onto slides, coverslipped with Ultra Cruz Mounting Medium, and sealed (nail polish). Digital images were taken with the following air objectives: 2X (0.06 NA), 10X (0.25 NA), and 20X (0.40NA).

Western Blots. Rats were given a bilateral injection of AQ and sacrificed at one of the following time points: 2 min, 10 min, 30 min, 1 hr, 12 hr, or 24 hr. Brains were removed, rapidly frozen, and stored at -80°C. The dorsal HPC and somatosensory cortex were dissected out and homogenized separately. Samples were centrifuged and the supernatant removed and measured using a Bradford protein assay kit (Bio-Rad). Protein samples were normalized and loaded for SDS-PAGE (9%). Proteins were transferred onto membranes using a semidry transfer apparatus (Bio-Rad). Membranes were then incubated in blocking buffer (2 hr), primary antibody (overnight at 4 °C; 1:200 mouse anti-aequorin [Chemicon], and secondary antibody (90 min; 1:5000 anti-mouse [Santa Cruz Biotechnology]). Membranes were then washed, placed in a chemiluminescence solution (Santa Cruz Biotechnology), and exposed to autoradiographic film (Hyperfilm MP). Images were taken and densitometry was performed using NIH Image J Software. A percentage of control score was derived for each rat by dividing each animal's relative optical density score by the somatosensory cortex control mean.

RESULTS - EFFECTS OF AQ INJECTIONS ON CELL DEATH

1. Aequorin protects hippocampal neurons against ischemic cell death

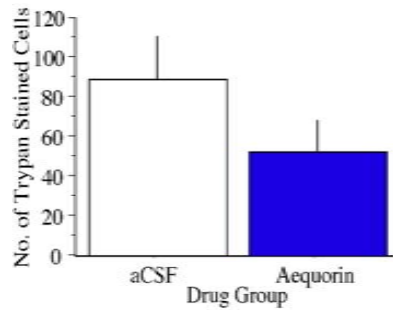
A. Control



B. Aequorin

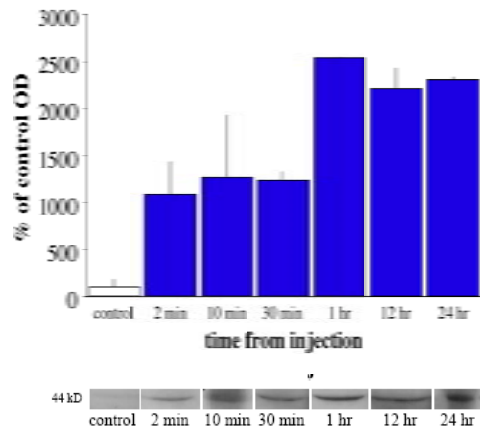


C. Summary of Adult Data



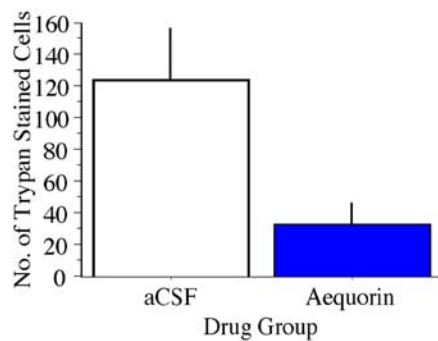
RESULTS – AQ WESTERN BLOTS

2. Time course of AQ detection in dorsal hippocampus



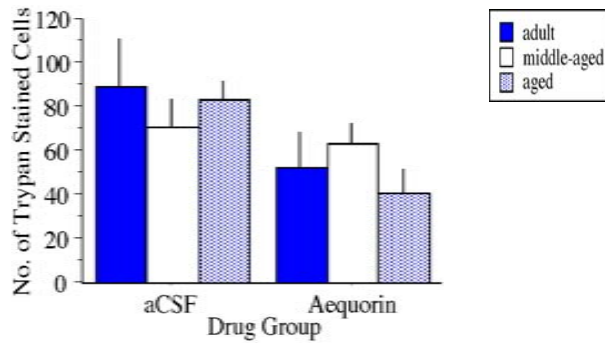
RESULTS – EXTRACELLULAR AQ

3. AQ injected without DMSO is also neuroprotective



RESULTS - EFFECTS OF AQ ON CELL DEATH AS A FUNCTION OF AGE

4. Aequorin protects hippocampal neurons regardless of age



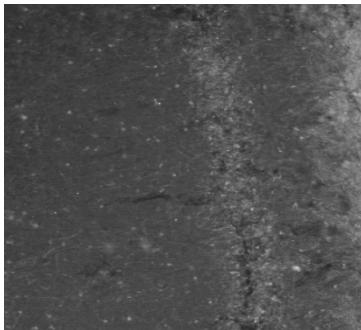
Age Group	Mean Age	No. of Rats	No. Trypan Blue Cells	
			aCSF (n)	AQ (n)
Adult	5.9 ± 0.2 mo	6	89.4 ± 21.0 (7)	52.2 ± 15.8 (13)
Middle-Aged	15.8 ± 0.6 mo	14	70.7 ± 12.2 (22)	63.4 ± 8.9 (25)
Aged	25.3 ± 0.3 mo	8	83.3 ± 8.6 (24)	41.1 ± 10.8 (14)

n = number of slices exposed to either control- (aCSF) or aequorin-treatment (AQ)

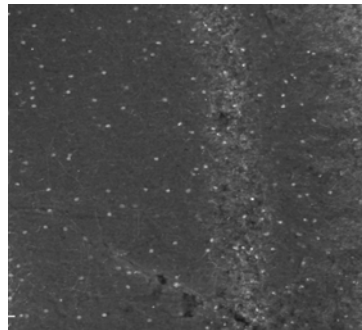
RESULTS - ACTIVE CASPASE-3 LABELING

5. *In vitro* model of ischemia induces apoptosis as measured by active caspase-3 in area CA1 of hippocampus

A. No ischemia



B. 5 min ischemia



SUMMARY

1. Injection of aequorin prior to ischemia is neuroprotective

- There were fewer dead cells in area CA1 of hippocampus in the AQ-injected rats compared to control-injected rats.
- Studies are under way to compare the effectiveness of extracellular versus intracellular AQ treatment. When AQ was injected without DMSO (which is normally used to facilitate uptake of AQ by neurons), there were also fewer dead cells in the AQ-injected animals compared to control-injected animals.

2. Aequorin is neuroprotective in adult, middle-aged, and aged animals

- The neuroprotective effects of AQ may be greatest in aged tissue.

3. The *in vitro* model of ischemia results in apoptosis

- There were more active caspase-3 labeled cells in the slices that received ischemia compared to control slices that were not subjected to ischemia.
- Future studies will attempt to determine whether AQ-treatment protects neurons by inhibiting apoptosis.

REFERENCES

- Ankarcrona, M., Dypbukt, J. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A., et al. (1995). Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron*, 15(4), 961-973.
- Baimbridge, K. G., Celio, M. R., & Rogers, J. H. (1992). Calcium-binding proteins in the nervous system. *Trends in Neuroscience*, 15(8), 303-308
- Bano, D., Young, K. W., Guerin, C. J., Lefevre, R., Rothwell, N. J., Naldini, L., et al. (2005). Cleavage of the plasma membrane Na⁺/Ca²⁺ exchanger in excitotoxicity. *Cell*, 120(2), 275-285.
- Chard, P. S., Bleakman, D., Christakos, S., Fullmer, C. S., & Miller, R. J. (1993). Calcium buffering properties of calbindin D28k and parvalbumin in rat sensory neurones. *Journal of Physiology*, 472, 341-357.
- Choi, D. W. (1992). Excitotoxic cell death. *Journal of Neurobiology*, 23(9), 1261-1276.
- Cobbold, P. H., & Lee, J. A. C. (1991). Aequorin measurements of cytoplasmic free calcium. In P. H. Cobbold (Ed.), *Cellular calcium: a practical approach* (pp. 55-81). New York: Oxford University Press.
- DeRenzis, F. A., & Schechtman, A. (1973). Staining by neutral red and trypan blue in sequence for assaying vital and nonvital cultured cells. *Stain Technology*, 48(3), 135-136.
- De Jong, G. I., Naber, P. A., Van der Zee, E. A., Thompson, L. T., Disterhoft, J. F., & Luiten, P. G. M. (1996). Age-related loss of calcium binding proteins in rabbit hippocampus. *Neurobiology of Aging*, 17(3), 459-465.
- Fan, Y., Shi, L., Gu, Y., Zhao, Y., Xie, J., Qiao, J., et al. (2007). Pretreatment with PTD-calbindin D 28k alleviates rat brain injury induced by ischemia and reperfusion. *Journal of Cerebral Blood Flow and Metabolism*, 27(4), 719-728.
- Gary, D. S., Sooy, K., Chan, S. L., Christakos, S., & Mattson, M. P. (2000). Concentration- and cell type-specific effects of calbindin D28k on vulnerability of hippocampal neurons to seizure-induced

injury. *Brain Research. Molecular Brain Research*, 75(1), 89-95.

Hof, P. R., & Morrison, J. H. (1991). Neocortical neuronal subpopulations labeled by a monoclonal antibody to calbindin exhibit differential vulnerability in Alzheimer's disease. *Experimental Neurology*, 111, 293-301.

Iacopino, A. M., & Christakos, S. (1990). Specific reduction of calcium-binding protein (28 kilodalton calbindin-D) gene expression in aging and neurodegenerative diseases. *Proceedings of the National Academy of Sciences (USA)*, 87, 4078-4082.

Kirino, T., & Sano, K. (1984). Selective vulnerability in the gerbil hippocampus following transient ischemia. *Acta Neuropathologica*, 62(3), 201-208.

Kristian, T., & Siesjo, B. K. (1998). Calcium in ischemic cell death. *Stroke*, 29(3), 705-718.

Krzywkowski, P., Potier, B., Billard, J. M., Dutar, P., & Lamour, Y. (1996). Synaptic mechanisms and calcium binding proteins in the aged rat brain. *Life Sciences*, 59(5/6), 421-428.

Lee, J. M., Zipfel, G. J., & Choi, D. W. (1999). The changing landscape of ischaemic brain injury mechanisms. *Nature*, 399(6738 Suppl), A7-14.

Lin, R. C., & Scheller, R. H. (2000). Mechanisms of synaptic vesicle exocytosis. *Annual Review of Cell and Developmental Biology*, 16, 19-49.

Mattson, M. P., & Magnus, T. (2006). Ageing and neuronal vulnerability. *Nature Reviews. Neuroscience*, 7(4), 278-294.

Mikkonen, M., Alafuzoff, I., Tapiola, T., Soininen, H., & Nettiinen, R. (1999). Subfield- and layer-specific changes in parvalbumin, calretinin and calbindin-D28K immunoreactivity in the entorhinal cortex in Alzheimer's disease. *Neuroscience*, 92(2), 515-532.

Moyer, J. R., Jr., & Brown, T. H. (1998). Methods for whole-cell recording from visually preselected neurons of perirhinal cortex in brain slices from young and aging rats. *Journal of Neuroscience Methods*, 86(1), 35-54.

Moyer, J. R., Jr., & Brown, T. H. (2006). Impaired trace and contextual fear conditioning in aged rats. *Behavioral Neuroscience*, 120(3), 612-624.

Moyer, J. R., Jr., S. M. Kelsey, McGann, J. P., & Brown, T. H. (2001). Morphology and distribution of calbindin-D28k in adult and aged rat perirhinal cortex. *Society for Neuroscience Abstracts*. 27, program number 327.7.

Sutherland, M. K., Wong, L., Somerville, M. J., Yoong, L. K. K., Bergeron, C., Parmentier, M., et al. (1993). Reduction of calbindin-28k mRNA levels in Alzheimer as compared to Huntington hippocampus. *Brain Research. Molecular Brain Research*, 18(1-2), 32-42.

Toma, S., Chong, K. T., Nakagawa, A., Teranishi, K., Inouye, S., & Shimomura, O. (2005). The crystal structures of semi-synthetic aequorins. *Protein Science*, 14(2), 409-416.

Ueda, H., & Fujita, R. (2004). Cell death mode switch from necrosis to apoptosis in brain. *Biological and Pharmaceutical Bulletin*, 27(7), 950-955.

Villa, A., Podini, P., Panzeri, M. C., Racchetti, G., & Meldolesi, J. (1994). Cytosolic Ca²⁺ binding proteins during rat brain ageing: loss of calbindin and calretinin in the hippocampus, with no change in the cerebellum. *European Journal of Neuroscience*, 6, 1491-1499.

West, A. E., Chen, W. G., Dalva, M. B., Dolmetsch, R. E., Kornhauser, J. M., Shaywitz, A. J., et al. (2001). Calcium regulation of neuronal gene expression. *Proceedings of the National Academy of Sciences (USA)*, 98(20), 11024-11031.

Yenari, M. A., Minami, M., Sun, G. H., Meier, T. J., Kunis, D. M., McLaughlin, J. R., et al. (2001). Calbindin d28k overexpression protects striatal neurons from transient focal cerebral ischemia. *Stroke*, 32(4), 1028-1035.

Zipfel, G. J., Babcock, D. J., Lee, J. M., & Choi, D. W. (2000). Neuronal apoptosis after CNS injury: the roles of glutamate and calcium. *Journal of Neurotrauma*, 17(10), 857-869.

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