HHS Public Access

Author manuscript

Anesth Analg. Author manuscript; available in PMC 2022 August 17.

Published in final edited form as:

Anesth Analg. 2010 December ; 111(6): 1400–1410. doi:10.1213/ANE.0b013e3181f7181c.

The Cytoprotective Effects of Dantrolene: A Ryanodine Receptor Antagonist

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Abstract

Calcium, as a second messenger, has an important role in a variety of cellular functions. However, disruption of intracellular calcium homeostasis leads to cytotoxicity and cell death. Excessive calcium release from intracellular stores, via the calcium channel ryanodine receptor, contributes to cell damage. Dysfunction of calcium homeostasis is established in tissue culture and animal models of ischemia, hypoxia, seizure, trauma, anesthesia, and neurodegenerative diseases. Dantrolene, the primary drug to treat malignant hyperthermia, is a ryanodine receptor antagonist. Dantrolene inhibits abnormal calcium release from the sarco-endoplasmic reticulum, which is the primary intracellular calcium store. Dantrolene has been investigated widely for its possible cytoprotective effects against cell damage in different tissue culture or animal models of diseases involving cytotoxicity induced by disruption of intracellular calcium homeostasis in pathogenesis. In this review, we summarize the role of the disruption of intracellular calcium homeostasis on cell death, the pharmacologic and pharmaco-kinetic features of dantrolene, and the cytoprotective effects and potential application of dantrolene for the inhibition of cell damage in a wide variety of models of stress and disease.

Dantrolene sodium, a ryanodine receptor (RYR) antagonist, is very well known in anesthesiology practice. The RYR is one of 2 major calcium release channels; the other is the inositol-1,4,5-trisphosphate receptor ($InsP_3R$). Dantrolene has been in clinical use since the 1980s for treating malignant hyperthermia (MH) and more recently for neuroleptic malignant syndrome,[1] spasticity,[2,3] heat stroke,[4] and ecstasy intoxication.[5] The cytoprotective effects of dantrolene are currently being investigated. This review summarizes the pharmacology of dantrolene, the role of calcium in cytotoxicity, and the cytoprotective role of dantrolene on cell damage induced by a wide variety of stress factors.

Dantrolene was synthesized by Snyder et al.[6] in 1967 as a new class of skeletal muscle relaxant. Animal studies showed that dantrolene inhibited the twitch response by direct action on the muscle. It dissociates the excitation-contraction coupling in muscle by

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AUTHOR CONTRIBUTIONS

SI helped write the manuscript; HW helped in mentoring. Both authors approved the final manuscript.

Disclosure: The authors report no conflicts of interest.

inhibiting the release of calcium (Ca^{2+}) from the sarcoplasmic reticulum (SR) but has no effect on the electrical excitability of the muscle, or the neuromuscular junction.[7,8]

The effectiveness of dantrolene on MH was reported first in swine that were susceptible to halothane anesthesia and presented clinical symptoms similar to human MH.[9,10] Results of a multicenter study performed between 1977 and 1979 in patients showed that the use of dantrolene for treatment of MH decreased the mortality rate significantly[11] and, since then, the mortality rate has decreased from 80% to <5% today.[12]

PHARMACOLOGY OF DANTROLENE

Dantrolene sodium (1-[[[5-(4-nitrophenyl)-2-furanyl]-methylene]amino]-2,4-imidazolidinedione sodium salt), a hydantoin derivative, is highly lipophilic and poorly soluble in water. Vials include 20 mg dantrolene and 3000 mg mannitol for IV administration. A vial is dissolved in 60 mL water and pH is adjusted to 9.5 using sodium hydroxide. The solution is light sensitive, so it should be protected from light in room temperature and should be used within 6 hours.

Dantrolene inhibits RYRs. These receptors are expressed on the surface of the endoplasmic reticulum (ER) and SR.[13,14] RYRs release Ca^{2+} from intracellular stores in the ER and SR.[15,16] Three different isoforms of RYRs are identified in mammalian tissues. RYR1 is mainly expressed on the terminal cisternae of SR in skeletal muscle.[13] RYR2 is primarily expressed in cardiac muscle[14] and dantrolene has no marked effect on this receptor.[17] In cardiac muscle, membrane depolarization causes opening of $Ca_v1.2$ L-type calcium channels, which leads to influx of Ca^{2+} from extracellular space. As a result of Ca^{2+} influx, RYR2 is stimulated and Ca^{2+} is released from the SR.[18]

RYR3 is expressed in low levels in most tissues; however, it is found most abundantly in the brain.[19,20] All 3 RYR isoforms are expressed in the central nervous system. RYR1 is found in cerebellar Purkinje cells and RYR2 is the predominant form in the olfactory nerve layer, cerebral cortex, dentate gyrus, cerebellar granule cells, the motor trigeminal nucleus, and the facial nucleus. RYR3 is high in the hippocampal CA1 pyramidal layer, caudate putamen, and dorsal thalamus.[19,20] Dantrolene acts directly on RYR1 and RYR3 to inhibit the extent of channel activation by calmodulin (CaM) and decreases the Ca^{2+} sensitivity of channel activation.[21]

In healthy volunteers, a 5-hour IV administration of dantrolene with an accumulative dose of 2.4 mg/kg caused a 75% depression in muscle twitch response. The plasma dantrolene concentration was 4.2 μg/mL and the elimination half-life was 12 hours. The plasma concentration was maintained in the therapeutic range for approximately 5 hours.[22] After oral administration of dantrolene, 70% is absorbed, peak plasma levels are obtained 4 to 6 hours later, and the elimination half-life is 8.7 hours.[2]

Dantrolene is metabolized in the liver by oxidative and reductive pathways. Through the oxidative pathway, the hydantoin ring is hydroxylated and forms 5-hydroxydantrolene, and through the reductive pathway, nitro-moiety and acetylation of the benzene ring forms the reduced acetylated derivative of dantrolene. Dantrolene is excreted in both urine and bile;

79% as 5-hydroxydantrolene, 17% as reduced acetylated derivative, and 4% as the main compound.[23]

Dantrolene is the primary drug used for the treatment of MH. MH is a pharmacogenetic disorder of skeletal muscle in humans and other vertebrates. Halogenated or depolarizing anesthetics trigger dysregulated release of Ca^{2+} from the SR and cause sustained activation of the contractile apparatus, muscular rigidity, and hyperthermia. Mutations in RYR1 result in an excessive Ca^{2+} release from the SR by direct activation of the channel by halogenated volatile anesthetics in MH. Halothane selectively activates mutated but not wild-type RYR1.[24,25] Halothane (as a trigger for Ca^{2+} release) is used to test MH susceptibility for an accepted, standard caffeine-halothane contracture test for the laboratory diagnosis of MH.[26,27] During excitation-contraction coupling in skeletal muscle, an action potential initiated at the neuromuscular junction rapidly transmits down the surface and T tubule membranes, which causes voltage-driven conformational changes in the T tubule dihydropyridine or voltage sensor L-type calcium channel ($Ca_v1.1$). This results in a direct mechanical interaction between L-type calcium channel and RYR1 and releases Ca^{2+} from the SR.[18,28] A recent study showed that within the clinical range, halothane did not induce Ca^{2+} release from SR in fibers obtained from normal human skeletal muscle. However, halothane elicited Ca^{2+} release from the SR, led to depletion of Ca^{2+} in the store, and triggered store-operated calcium influx in MH-susceptible human skeletal muscle fibers.[29] Depletion of Ca^{2+} from intracellular stores activates store-operated calcium influx or capacitative Ca^{2+} entry and causes Ca^{2+} entry across plasma membrane.[30] These results emphasize the importance of RYR1 receptor antagonists for treatment of MH.

Azumolene (1-[[[5-(4-bromophenyl)-2-oxazoly]methylene] amino]-2,4-imidazolidinedione), an analog of dantrolene, is a potential alternative drug for treating MH. It is 30-fold more water soluble than dantrolene.[31] Azumolene was synthesized by replacing the *para*-nitro-phenyl group in dantrolene sodium with a *para*-bromo-phenyl group.[32] Chemical structures of dantrolene and azumolene are shown in Figure 1. Azumolene suppresses the opening rate of RYR Ca^{2+} release channels within skeletal fibers.[33] Azumolene reverses MH episodes induced by halothane in susceptible swine[31,34] and inhibits caffeine-induced contractions in normal and human MH-susceptible skeletal muscle with a potency similar to dantrolene.[35]

The most common side effects of dantrolene with IV and chronic oral administration are dizziness, drowsiness, light headedness, headaches, anorexia, diarrhea, nausea, and vomiting.[2,22] Chronic oral use can be associated with liver dysfunction.[36] Rarely observed side effects are fatigue, weakness, rash, and acne-like dermatosis.[2,37] Chronic pleural effusion was also reported during dantrolene use in patients.[38] During IV administration, extravasation of dantrolene can cause thrombophlebitis (due to basic pH); therefore, it is suggested to use large veins for injection.

Coadministration of dantrolene with other drugs also produces side effects. Dantrolene given with verapamil leads to an increase in cardiac dysfunction in swine and dogs.[39,40] Although this effect has not been described in humans, it is recommended not to use this combination during MH in humans; correction of acidosis and hyperkalemia will be more helpful for treating arrhythmias. Driessen et al.[41] reported that recovery time from neuromuscular

blockade induced by vecuronium was longer in patients treated with dantrolene for MH prophylaxis compared with normal patients.

Although the common side effects of dantrolene originate in the central nervous system, it is still controversial whether dantrolene passes through the blood-brain barrier (BBB). Wuis et al.[42] reported a high accumulation of radioactivity in the intestines, liver, gall bladder, kidneys, and urinary bladder but not in the brain of marmoset monkeys treated with ^{14}C-dantrolene. Enokizono et al.[43] showed that breast cancer resistance protein (Bcrp), a member of the adenosine triphosphate (ATP)-binding cassette protein transporter family, limits the tissue penetration of dantrolene, as well as some other xenobiotic compounds in the BBB in mice. Distribution and brain uptake of dantrolene were significantly increased in Bcrp knockout mice compared with wild-type littermates. There is also evidence to support the penetration of dantrolene across the BBB. First, dantrolene is a small molecule (molecular weight: 399) and has high lipid solubility. Second, it has central side effects (drowsiness and dizziness), and alters neurotransmitter levels in the cerebrospinal fluid.[44,45] A dose sufficient to cause muscle relaxation may be inadequate to produce a sufficiently high concentration of active agent in the brain, because the pharmacologic effects of dantrolene are dose dependent in most models.[45]

ROLE OF CALCIUM IN CYTOTOXICITY AND NEURODEGENERATION

Ca^{2+}, as a second messenger, has an important role in a variety of cellular functions such as control of cell growth and differentiation, membrane excitability, exocytosis, synaptic activity, apoptosis, and autophagy.[46 – 48] The intracellular free Ca^{+2} is highly regulated and its concentration is maintained at approximately 100 nM. Under normal conditions, there is a 10,000-fold Ca^{2+} gradient between the intra- and extracellular space.[46] Thus, small or localized increases in intracellular Ca^{2+} will quickly trigger physiologic events such as the activation of enzymes or ion channels.

An increase in intracellular Ca^{2+} can arise from influx from the extracellular space through voltage-dependent calcium channels (VDCCs), receptor-operated calcium channels, and store-operated calcium channels, which are located on the plasma membrane or from the release of Ca^{2+} from intracellular stores such as the ER, SR, or mitochondria. Ca^{2+} levels in the ER are regulated by sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps; inositol-1,4,5-triphosphate ($InsP_3$) and $InsP_3$Rs; RYRs; and Ca^{+2}-binding proteins (calreticulin and calsequestrin). In unstimulated cells, normal cytosolic Ca^{+2} is regulated (to concentrations <100 nm) by uptake into the ER by SERCA and by efflux into the extracellular space either through the plasma-membrane Ca^{2+}-ATPase or the Na^+/Ca^{2+} exchanger, both of which are located on plasma membrane.[46] Ca^{2+} release from the ER is triggered via agonist activation of $InsP_3$ Rs by $InsP_3$, which is generated through the phospholipase C pathway[49] and by activation of RYRs. Mitochondria store Ca^{2+} electrophoretically by a uniport transporter and release Ca^{2+} via reversal of the uniporter, Na^+/H^+-dependent Ca^{2+} exchange, or the permeability transition pore. Mitochondria rapidly take up Ca^{2+} released from the ER. Close contact between the ER/SR and mitochondria has been observed by electron microscopy in several fixed cell types.[50] The ER and mitochondria are linked through ER-mitochondrial–associated membranes. This

area is rich in enzymes and proteins that are involved in lipid biosynthesis and Ca^{2+} signaling. Mitochondria preferentially accumulate Ca^{2+}in cytosolic areas rich in Ca^{2+}, called microdomains.[51] Furthermore, enriched $InsP_3R$ immunoreactivity has been found in ER regions close to mitochondria.[50]

The ER has 2 major functions in the cell: Ca^{2+} storage and the facilitation of the proper folding of newly synthesized proteins destined for secretion to the cell surface or other intracellular organelles. ER stress occurs in a variety of physiologic and pharmacologic situations, for example, when the capacity of the ER to fold proteins becomes saturated as a result of expression of folding-incompetent or aggregation-prone proteins, when there is overload or depletion of the ER Ca^{2+} pool, and with glucose starvation and hypoxia.[47] During ER stress, 2 pathways are activated to remove the incorrectly folded and/or accumulated proteins. Stimulation of the unfolding protein response pathway induces ER chaperones, such as immunoglobulin heavy-chain binding protein, glucose-regulated protein-78, calreticulin, protein disulfide isomerase, and the transcription factor CHOP/GADD153. Stimulation of the ER overload response pathway causes the production of cytokines and interferons through activation of nuclear factor-κB.[46] Prolonged ER stress can induce apoptosis to eliminate the damaged cells.

One of the consequences of Ca^{2+} overload is neurodegeneration, a progressive loss of structure and function as well as death of neurons. As early as the 1970s, researchers reported an association between an increase in intracellular Ca^{2+} concentration [Ca^{2+}] and neurotoxicity. Schlaepfer and Bunge[52] investigated the degenerative changes in amputated nerve fibers in cultured rat sensory ganglia in the presence of media containing different concentrations of Ca^{2+}. They observed loss of neurofilaments and microtubules in association with high concentrations of Ca^{2+}. Choi[53] reported that glutamate, the major excitatory amino acid in the brain, induced toxicity as a result of increased [Ca^{2+}] in neurons in cell culture. Glutamate is released from presynaptic vesicles and binds to postsynaptic glutamate receptors. Three classes of glutamate ionotropic receptors are *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (KA). The opening of glutamate-gated channels leads to Ca^{2+} influx from the extracellular space into the cytosol, which in turn stimulates Ca^{2+} release (calcium-induced calcium release [CICR]) from intracellular stores. CICR is also activated by other voltage-gated calcium channel–induced Ca^{2+} influx into the cytosol. CICR is primarily mediated by RYR and Ca^{2+} release from the ER and SR.[54] Furthermore, NMDA and KA caused reversible and irreversible neuronal damage[55] and these neurotoxic effects depended on [Ca^{2+}] in rat cerebellar slices.[56]

Increased cytosolic Ca^{2+} also induces a secondary Ca^{2+}-dependent phenomena, which contributes to neurotoxicity and cell death through apoptosis by activating proteases, lipases, and endonucleases. When Ca^{2+} binds to CaM, it activates nitric oxide synthase and increases the formation of nitric oxide. Free oxygen radicals react with nitric oxide and form peroxynitrite, a highly toxic molecule that damages DNA and proteins. DNA cleavage activates the DNA repair enzyme, poly (ADP-ribose) polymerase, which requires ATP for functioning. Decreased ATP levels cause the formation of oxygen radicals in mitochondria and increases cellular damage. Mitochondrial permeability is increased via

activation of the mitochondrial permeability transition pore and results in osmolar load inside the mitochondria, causing mitochondrial swelling and rupture as well as the release of cytochrome *c* (CytC). CytC activates pro-apoptotic factors. Protease activation also leads to cytoskeletal breakdowns. The stimulation of phospholipase A_2 releases arachidonic acid and related polyunsaturated fatty acids. During fatty acids metabolism, reactive oxygen species are also generated.[46,57,58] This cascade of events leads to apoptosis and neurotoxicity.

Recent studies also indicate that cytosolic Ca^{2+} is involved in autophagy, a lysosomal degradation pathway to eliminate cellular proteins and organelles. Høyer-Hansen et al.[48] demonstrated that different Ca^{2+} mobilizing agents such as vitamin D3, ionomycin, ATP, and thapsigargin induce autophagy by inhibiting the autophagy inhibitory activity of rafamycin via activation of Ca^{2+}/CaM-dependent kinase kinase-β and AMP-activated protein kinase. Inhibition of $InsP_3Rs$ using a pharmacologic inhibitor (xestospongin B), siRNA depletion, or lithium-mediated inhibition of $InsP_3$ synthesis triggers autophagosome formation, suggesting that stimulation of $InsP_3Rs$ inhibits autophagy.[59,60]

CYTOPROTECTIVE EFFECTS OF DANTROLENE

Effects of Dantrolene on Ischemia-Induced Cytotoxicity

Excessive presynaptic glutamate release occurs during hypoxia and ischemia.[61,62] Cerebral ischemia can significantly increase the extracellular concentration of excitatory amino acids and cause neuronal membrane depolarization, which results in Ca^{2+} influx from the extracellular space into the cytosol.[63,64] Mitani et al.[65] demonstrated that approximately one-third of the increased [Ca^{2+}] after ischemic stimulation was derived from an extracellular component and the remaining two-thirds was derived from intracellular stores in hippocampal slices. They also showed that administration of dantrolene diminished by half the [Ca^{2+}] mobilization from intracellular stores. Zhang et al.[66] reported that intracerebroventricular injection of dantrolene protected neurons from ischemic, delayed neuronal death induced by bilateral occlusion of the common carotid artery in the gerbil brain. Wei and Perry[45] demonstrated that pretreating hypothalamic neurosecretory cells with dantrolene significantly reduced the increase in cytosolic [Ca^{2+}] and neuronal death observed after those cells were exposed to thapsigargin (an ER Ca^{2+}-ATPase) alone. Furthermore, the authors reported that dantrolene administered systemically before induction of global cerebral ischemia in gerbils significantly increased the number of intact CA1 pyramidal neurons in a dose-dependent manner. Dantrolene ameliorates neuronal cell death induced by transient ischemia in rats[67,68] as well as in neuronal cell lines exposed to hypoxia and glucose deprivation.[69] Moreover, infarct size after ischemia was also reduced by injection of dantrolene during reperfusion in adult[70] and neonatal rats.[71] Kocogullari et al.[72] studied whether dantrolene would be protective against neuronal injury during aortic ischemia/reperfusion (mimicking spinal cord injury after aortic surgical intervention) in rabbits. Dantrolene administered intraperitoneally (IP) just 30 minutes before the surgery significantly improved neurologic deficits and decreased vascular proliferation, hemorrhage, edema, and neuron loss in spinal cord sections. Dantrolene has been shown to have synergistic effects with nimodipine, a voltage-dependent L-type Ca^{2+} channel blocker, against serotonin-induced vasoconstriction in isolated cerebral arteries of rats.[73]

Also, dantrolene inhibited presser responses induced by noradrenaline, angiotensin, and endothelin-1 in isolated arterial preparations from rodents.[74–76]

However, Martínez-Sánchez et al.[77] found that dantrolene was not effective in preventing neuronal loss in hippocampal slice cultures exposed to oxygen-glucose deprivation. They concluded that oxygen-glucose deprivation–induced cell death is mediated by activation of ionotropic glutamate receptors, voltage-dependent Na^+ channels, and both plasma membrane and mitochondrial Na^+/Ca^{2+} exchangers. In in vivo and in vitro models of myocardial reperfusion injury, dantrolene reduced creatinine kinase release (measured as an index of cell death) during reperfusion of isolated rat hearts, but did not have any effect on infarct size or hemodynamics during reperfusion after myocardial ischemia in rabbits.[78] Another study, reported by Wu et al.[79] also demonstrated that dantrolene did not protect renal function against ischemia/reperfusion injury in cell cultures and in rats. Both nicardipine, an L-type calcium channel inhibitor, and TMB-8, an $InsP_3R$ antagonist, inhibited CytC release and caspase-3 activation, and decreased the apoptotic cell number suggesting an important role of the $InsP_3R$ on ER or SR membrane cytotoxicity. In addition, pretreating rats with these 2 compounds, but not dantrolene, significantly prevented an increase in serum creatinine levels during the ischemia/reperfusion injury.

Effects of Dantrolene on Glutamate-Induced Cytotoxicity

Pretreating guinea pigs with intravitreal dantrolene and nimodipine protected retinal ganglion cells against intravitreal NMDA-induced retinal injury.[80] Dantrolene has also been shown to prevent KA-induced[81] and NMDA-induced[82] neuronal cell death in cerebellar granule cell cultures. After KA-induced seizures, RyR3 mRNA upregulation was observed in the hippocampal CA3 region and striatum, and c-fos mRNA expression increased in the hippocampus, dentate gyrus, and deeper layer of the neocortex.[83] Furthermore, IP administration of dantrolene before induction of seizures by IP injection of KA reduced the apoptotic cell death in the hippocampal CA1 region and parietal cortex in rats.[81] Makarewicz et al.[84] studied the mechanism behind this protection using radioactive Ca^{2+}. They demonstrated that dantrolene inhibited the NMDA-evoked ^{45}Ca uptake in cerebellar granule cell cultured neurons in a dose-dependent manner.

Ca^{2+} influx is essential for the first stage (intrinsic burst firing) of epileptic neuronal events.[85] Results from animal models of epilepsy have introduced the possibility that Ca^{2+} antagonists may be a new class of anticonvulsant agents.[86] Seizures induced by a metabotropic receptor agonist, 1*S*,3*R*-ACPD, were completely abolished with a high dose of dantrolene but not by MK-801 in rats. Dantrolene also significantly diminished 1*S*,3*R*-ACPD–induced increase in brain volume.[87] Niebauer and Gruenthal[88] reported that when dantrolene was administered just 30 minutes after electrogenic-induced status epilepticus, it was more protective to the entire hippocampus than when injected 140 minutes after seizures in rats. Both dantrolene and thapsigargin prevented seizure-induced cell death in hippocampal slices suggesting that Ca^{2+} release from ER stores also contributes to seizure-induced cell death.[89] Furthermore, dantrolene significantly reduced seizure activity in the EL mouse, a mutant strain susceptible to convulsive seizures.[90]

Dantrolene combined with LY 300164, an AMPA/kainate receptor antagonist, to treat electroshock-induced seizures in mice, impaired motor performance.[91] Conversely, dantrolene had no marked effect against seizures induced by electroshock[92] and those induced by 3,5-DHPG, a group I metabotropic glutamate receptor agonist,[93] in mice.

Effects of Dantrolene on Neurodegeneration in Neurodegenerative Diseases

Ca^{2+} signaling is crucial for maintaining normal neuronal functions such as membrane excitability, neurotransmitter release, cellular growth, differentiation, and cell death. Under resting conditions, cytosolic [Ca^{2+}] in neurons is maintained at approximately 200 nM.[94] Disruptions in Ca^{2+} homeostasis have been reported in neurodegenerative diseases including Alzheimer disease (AD),[95–97] Parkinson disease (PD),[98,99] Huntington disease (HD),[94,100] amyotrophic lateral sclerosis (ALS),[101,102] as well as spinocerebellar ataxias (SCAs).[103,104] Calcium homeostasis can be disrupted by alterations in Ca^{2+} buffering capacity, increased sensitivity to excitotoxicity, functional changes in both plasma and ER Ca^{2+} channels, as well as mitochondrial Ca^{2+} homeostasis. The models of cytotoxicity induced by Ca^{2+} dysregulation during excitotoxicity, neurodegenerative diseases, and general anesthesia are summarized in Figure 2.

Alzheimer Disease—AD is the most common idiopathic, progressive, neurodegenerative disease characterized by a progressive and irreversible loss of neurons in specific brain areas involved in learning and memory processes. The disorder gradually affects memory, learning abilities, and language skills; causes behavioral and personality changes; interferes with an individual's ability to achieve daily activities, and finally leads to death. AD is characterized by extracellular deposits of amyloid-β peptide (Aβ) that result from altered proteolytic processing of amyloid precursor protein, intracellular neurofibrillary tangles composed of hyper-phosphorylated tau protein deposits and the reduced number of synapses and neuronal loss.[105]

There is growing evidence suggesting that disruption of ER Ca^{2+} signaling has a role in the pathogenesis of AD. PC12 cells expressing mutant human presenilin-1 (PSEN1) are more susceptible to oxidative stress and changes in intracellular Ca^{2+} levels, as well as more vulnerable to the general anesthetic isoflurane-induced Ca^{2+} release from the ER and cell apoptosis.[106,107] The increase of Ca^{2+} in response to thapsigargin in mutated cells was prevented by pretreating cells with nifedipine and dantrolene.[108] MacManus et al.[95] reported that Aβ40 significantly increases $^{45}Ca^{2+}$ influx into rat cortical synaptosomes via activation of L- and N-type VDCCs, and also increases the amplitude of N- and P-type Ca^{2+} channel currents in rat cultured cortical neurons. However, Rovira et al.[96] found that, using the whole-cell patch-clamp recording in hippocampal CA1 pyramidal cells of mice, Aβ(25–35) acts on L-type Ca^{2+} channels but not Aβ40. All amyloids, and particularly Aβ42, increased intracellular Ca^{2+} in fluo-3-loaded SH-SY5Y cells, which lasted after the depletion of intracellular Ca^{2+} stores, indicating that both extracellular and intracellular Ca^{2+} sources contribute to this effect.[97] In human cortical neurons, Aβ oligomers and Aβ-derived diffusible ligands have high affinity to synaptic contacts and cellular membranes. Aβ oligomers caused cellular changes and activated mitochondrial death via the apoptotic pathway.[109] Using multiphoton imaging, Kuchibhotla et al.[110] measured Ca^{2+} levels in

cortical neurons in several transgenic mouse models of AD and found Ca^{2+} overload in neurites in older transgenic mice associated with proximity to plaques as well as loss of spinodendritic Ca^{2+} compartmentalization, which is critical for synaptic integration.

Theoretically, compounds that prevent or correct Ca^{2+} dysregulation would be useful for the treatment of AD and other neurodegenerative disorders. An NMDA receptor antagonist, memantine, has been approved by the Food and Drug Administration for the treatment of AD.[99] Orally administered memantine to triple-transgenic (3xTg-AD) mice for 3 months significantly ameliorated cognitive dysfunction and reduced the levels of insoluble Aβ and prefibrillar soluble oligomers.[111] RYRs are of interest in regulating Ca^{2+} dysregulation in in vivo and in vitro models for AD. Exposure of Aβ42 to rabbit skeletal SR vesicles resulted in RYR-mediated Ca^{2+} release, and exposure of lipid bilayers to Aβ42 resulted in an increased probability of channel openings.[112] Intracellular Ca^{2+} levels were increased in cells expressing the human PSEN1 L286V mutation. Aβ induced cell death in these cells but both dantrolene and nifedipine protected the cells against these adverse effects.[108] Imaizumi et al.[113] showed that Aβ induced DP5, a neuronal apoptosis-inducing gene, expression in cultured rat cortical neurons, and also demonstrated that both nifedipine and dantrolene blocked the Aβ-induced DP5 expression.

It has been suggested that the PSEN1 mutation increases vulnerability of neurons to cerebral ischemia and oxidative stress. This hypothesis was examined in PSEN1 mutant knockin mice and in neuronal cultures from PSEN1-deficient mice, respectively.[114,115] The size of cerebral infarct, caused by middle cerebral artery occlusion, was bigger in PSEN1 knockin mice compared with wild-type littermates. Researchers also showed that cultured cortical neurons from PSEN1 mutant mice were more sensitive to glucose deprivation and chemical hypoxia, had a more prominent increase of intracellular Ca^{2+}, and that pretreating cells with dantrolene prevented this cell damage.[114] Nakajima et al.[115] also demonstrated that neurons cultured from PSEN1-deficient mice were more vulnerable to hydrogen peroxide (H_2O_2) treatment compared with wild-type controls. However, they reported that whereas antioxidants, BAPTA AM (an intracellular Ca^{2+} chelator), and nifedipine protected cells against H_2O_2-induced death, an N-type VDCC blocker ω-conotoxin or dantrolene did not prevent cell death. Moreover, Lopez et al.[116] stated that cultured neurons from 3xTg-AD mice had increased intracellular Ca^{2+} levels and that the application of nifedipine and xestospongin C partially blocked this increase but blocking RYRs had no effect. Even though there are conflicting results, compounds that affect intracellular Ca^{2+} stores, particularly dantrolene, look promising for the future treatment of AD.

Huntington Disease—HD is an autosomal dominant, progressive neurodegenerative disorder. It is caused by an expansion of the polyglutamine tract in the N-terminal region of the protein huntingtin. This defect leads to loss of medium-sized spiny GABAergic projection neurons of the caudate nucleus and putamen of the basal ganglia.[117,118]

The type 1 isoform of the $InsP_3R$ is the major member of the $InsP_3R$ family in the central nervous system and it is mainly expressed in cerebellar Purkinje cells, the hippocampal CA1 region, caudate-putamen, and cerebral cortex.[31] $InsP_3R1$-deficient mice have severe ataxia, tonic-clonic seizures, and die by the weaning period.[119] Tang et al.[100] reported

that huntingtin-associated protein-1 binds to $InsP_3R1$ and forms a complex. This complex facilitates Ca^{2+} release in medium spiny striatal neurons in response to 3,5-DHPG, a selective mGluR1/5 agonist, suggesting an explanation for the dysfunction of cytosolic Ca^{2+} signaling in HD patients. In their later report, Tang et al.[120] demonstrated that the binding site is located on the C-terminal cytosolic region of the $InsP_3R1$ and the introduction of GFP-IC10 protein, using a viral vector, stabilizes Ca^{2+} signaling and protects medium spiny neurons from glutamate-induced apoptosis. Tang et al.[121] reported that membrane-permeable $InsP_3R$ blockers, 2-APB and enoxaparin, are neuroprotective in medium spiny neurons in HD mice. Mutated huntingtin protein in mouse striatal neurons renders these cells vulnerable to isoflurane-mediated Ca^{2+} release from the ER via the $InsP_3R$ and cell apoptosis.[107] To our knowledge, there has been no investigation of the possible cytoprotective effects of RYR antagonists on HD.

Parkinson Disease—PD is characterized by rigidity, resting tremor, bradykinesia, and postural instability. Pathologic findings are loss of dopaminergic neurons in the substantia nigra pars compacta and the formation of Lewy bodies, which are intracytoplasmic inclusion bodies composed of aggregates of α-synuclein.[99,122] It has been suggested that mitochondrial dysfunction is crucial in the pathogenesis of PD[122] as well as α-synuclein–induced excitotoxicity. Danzer et al.[123] showed that α-synuclein oligomers increased $[Ca^{2+}]$ and caspase-3 activity in primary cell cultures. Furthermore, Furukawa et al.[124] reported that whereas BAPTA AM protects cells, neither nifedipine nor ω-conotoxin protected cells against excitotoxicity induced by an α-synuclein mutation. Memantine and isradipine, an L-type Ca^{2+} channel inhibitor, are in phase II clinical trials for the treatment of PD.[99] No study has directly investigated the possible cytoprotective effects of dantrolene on PD.

Amyotrophic Lateral Sclerosis—ALS is characterized by progressive loss of spinal and cortical motor neurons. Clinical findings are progressive loss of muscle force, breathing capacity, swallowing difficulties, and limb spasticity. ALS is largely a sporadic disease and only 5% to 10% of patients have an autosomal dominant inheritance of mutations in the enzyme superoxide dismutase 1.[102] Excitotoxicity and Ca^{2+} dysregulation are factors in the pathogenesis of ALS. Activated microglia release proinflammatory factors and large amounts of glutamate, which activate the AMPA and NMDA receptors. Ca^{2+} influx increases and results in mitochondrial Ca^{2+} overload, mitochondrial swelling, and apoptosis. Mutant superoxide dismutase 1 also impairs mitochondrial function and handling Ca^{2+} overload.[99,102] Memantine and riluzole, antiglutamate drugs, are in phase II/III clinical trials for the treatment of ALS.[99] Rothstein and Kuncl[101] showed that dantrolene provided partial motor neuron protection in organotypic cultures after non-NMDA receptor stimulation with threo-hydroxyaspartate, selectively inhibited glutamate transport, and induced glutamate toxicity.

Spinocerebellar Ataxia—SCAs are autosomal dominant neurodegenerative diseases caused by an expansion of polyglutamine tracts either in the nuclear protein ataxin-1 (ATx1) or the cytosolic protein ataxin-2 (ATx2) or ataxin-3 (ATx3). SCA1 and SCA2 mainly affect cerebellar Purkinje cells. Patients usually present with progressive ataxia, dysarthria, ophthalmoplegia, slow eye movements, and peripheral neuropathy.[104] The areas

affected in SCA3 are the dentate gyrus, pontine nuclei, globus pallidus, subthalamic nucleus, substantia nigra, and spinocerebellar tract. Chen et al.[103] showed that mutant ATx3 binds to the $InsP_3R1$, sensitizes to $InsP_3$, and increases cytosolic Ca^{2+} levels in HEK293 cells compared with control cells. They further reported that oral administration of dantrolene to SCA3-YAC-84Q transgenic mice improved motor coordination and increased neuronal cell counts. A similar mechanism was shown for SCA2. Mutant ATx2 to 58Q associates with the $InsP_3R1$, and sensitizes to $InsP_3$ in planar lipid bilayers. Increased Ca^{2+} release induced by DHPG and more prominent cell death induced by glutamate were observed in 58Q Purkinje cell cultures compared with wild-type cell cultures. Furthermore, pretreating cells with dantrolene protected 58Q Purkinje cells against glutamate-induced cell death. Similarly, oral administration of dantrolene to transgenic mice reduced age-dependent motor deficits and Purkinje cell loss.[104]

Other Cytoprotective Effects of Dantrolene

Intracellular Calcium Dysregulation and Anesthesia Neurotoxicity—Previous and recent studies suggest that inhaled anesthetics, especially isoflurane, induce neurotoxicity via disruption of intracellular Ca^{2+} homeostasis. In brain cortical slices and in cell cultures, application of inhaled anesthetics induces Ca^{2+} release from the ER, increases cytosol and mitochondrial [Ca^{2+}], depletes ER Ca^{2+} stores, and elicits apoptosis.[125–128] Wei et al.[126] reported that dantrolene suppresses cytotoxicity induced by isoflurane in cells.

Recent studies from our laboratory demonstrated that inhaled anesthetic–induced neurotoxicity is a consequence of overactivation of $InsP_3Rs$ by anesthetics.[127,128] Using DT40 chicken B lymphocytes with total $InsP_3Rs$ knockout and their wild-type control cells, Yang et al.[127] showed that whereas isoflurane, sevoflurane, and desflurane induced cell damage in control cells, with isoflurane being most potent, they had no effect on $InsP_3Rs$ knockout cells. Furthermore, Zhao et al.[128] reported that (a) the isoflurane-induced increase in cytosolic [Ca^{2+}] and cell damage was diminished in $InsP_3Rs$ knockdown primary cortical and hippocampal neurons; (b) isoflurane activated β-site amyloid β precursor protein-cleaving enzyme by activating $InsP_3Rs$; and (c) early postnatal exposure of isoflurane caused transient memory and learning impairment in neonatal rats. Anesthesia-induced neurodegeneration was also shown in pups exposed to isoflurane during delivery.[129] General anesthetics at low concentration or for short duration may be neuroprotective via preconditioning,[130,131] whereas general anesthetics at high concentration or for prolonged exposure may be neurotoxic.[129,131] Both neuroprotective and neurotoxic effects of general anesthetics may be exerted via activation of the $InsP_3R$ with differential degrees.[106,107,127,128,132] Additionally, there is growing concern that anesthesia may contribute to delirium and postoperative cognitive dysfunction as well as the onset and progression of AD,[133,134] although further clinical studies are needed to confirm these initial findings.

Sepsis—Increase of intracellular [Ca^{2+}] is a critical event in the pathophysiology of endotoxemia and sepsis.[135–137] Using the cecal ligation and perforation (CLP) sepsis model in rats, Song et al.[135] demonstrated that perfused aortic strips from septic animals released more than 2 times the amount of Ca^{2+} compared with strips from control rats and that

dantrolene inhibited this Ca^{2+} release. Dantrolene also decreased metabolic disruptions during CLP-induced sepsis in rats and increased survival rate in *Escherichia coli*–induced sepsis in mice.[136] Furthermore, whereas dantrolene inhibited lipopolysaccharide-elicited production of interleukin-12 and interferon γ levels, neither verapamil nor diltiazem had a marked effect.[137]

Williams et al.[138] investigated the underlying mechanism for the sepsis-induced catabolic response in skeletal muscle using CLP-induced sepsis in rats. They found that sepsis increased myofilament and calpain release and dis-integrated Z bands. Pre- or postsurgery administration of dantrolene significantly inhibited myofilament release and Z band disruption. In a following study, sepsis induced the ubiquitin-proteosome proteolytic pathway in skeletal muscle, and treatment with dantrolene prevented activation of this pathway and reduced protein degradation in rats.[139] Moreover, Hassoun et al.[140] demonstrated that the disruption of the SR to store Ca^{2+} and an increase in intracellular [Ca^{2+}] and mitochondrial dysfunction were a consequence of Ca^{2+} overload in lipopolysaccharide-induced sepsis in rats. Treatment with dantrolene decreased the Ca^{2+} overload and mitochondrial dysfunction.

Trauma—Aslan et al.[141] investigated the protective effects of dantrolene on a surgical spinal cord injury model in rabbits. Systemically administered dantrolene not only improved motor paralysis (at 24 hours) but also augmented antioxi-dative defense systems and decreased injury-induced apoptosis in spinal cord.

CONCLUSIONS

This review summarizes scientific studies investigating the possible cytoprotective effects of dantrolene on cytotoxicity mainly induced by deregulation in Ca^{2+} homeostasis. More studies are required to determine whether dantrolene is an effective cytoprotective drug in humans. Nevertheless, dantrolene has an advantage over other experimental compounds that regulate Ca^{2+} homeostasis, because it has long been in clinical use.

ACKNOWLEDGMENTS

The authors thank Drs. Roderic Eckenhoff and Maryellen Eckenhoff from the Department of Anesthesiology and Critical Care, University of Pennsylvania, Philadelphia, for valuable comments and editing of this review.

STUDY FUNDING

Supported by the National Institute of General Medical Science, National Institutes of Health, Baltimore, MD, K08 grant (1-K08-GM-073224, to HW) and R01 grant (1-R01GM084979-01, 3R01GM084979-02S1 to HW); and March of Dimes Birth Defects Foundation research grants (12-FY05-62 and 12-FY08-167 to HW), White Plains, NY.

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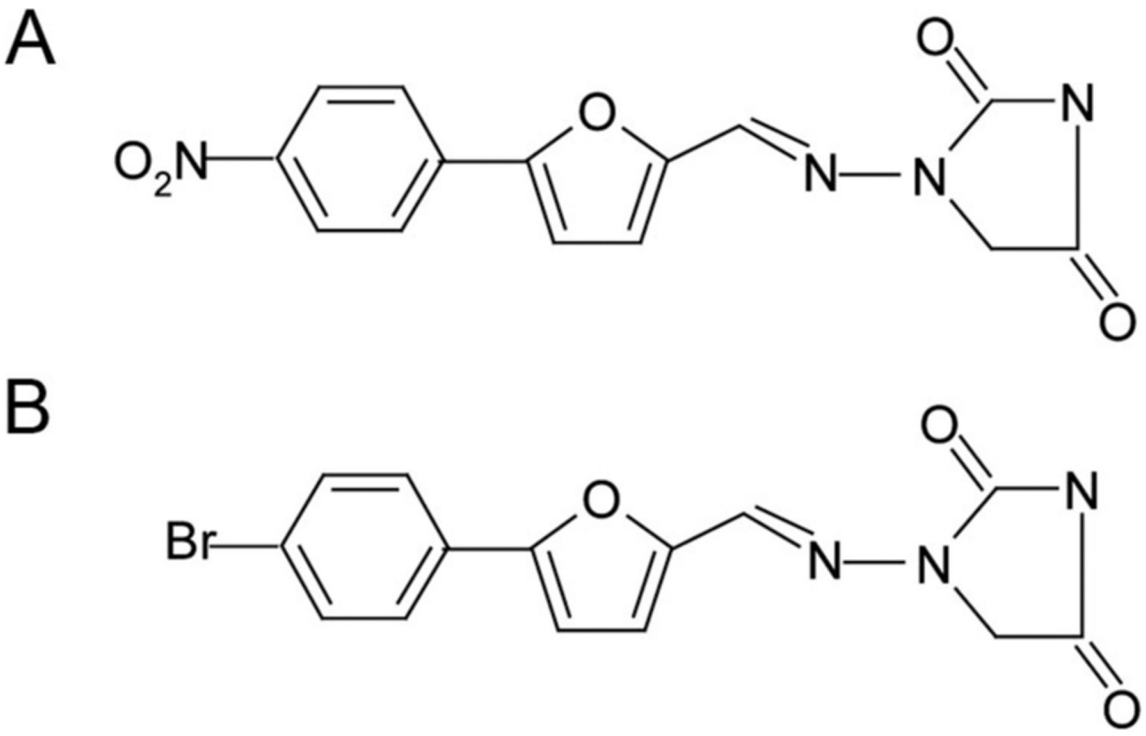

Figure 1.
Chemical structures of dantrolene (A) and azumolene (B).

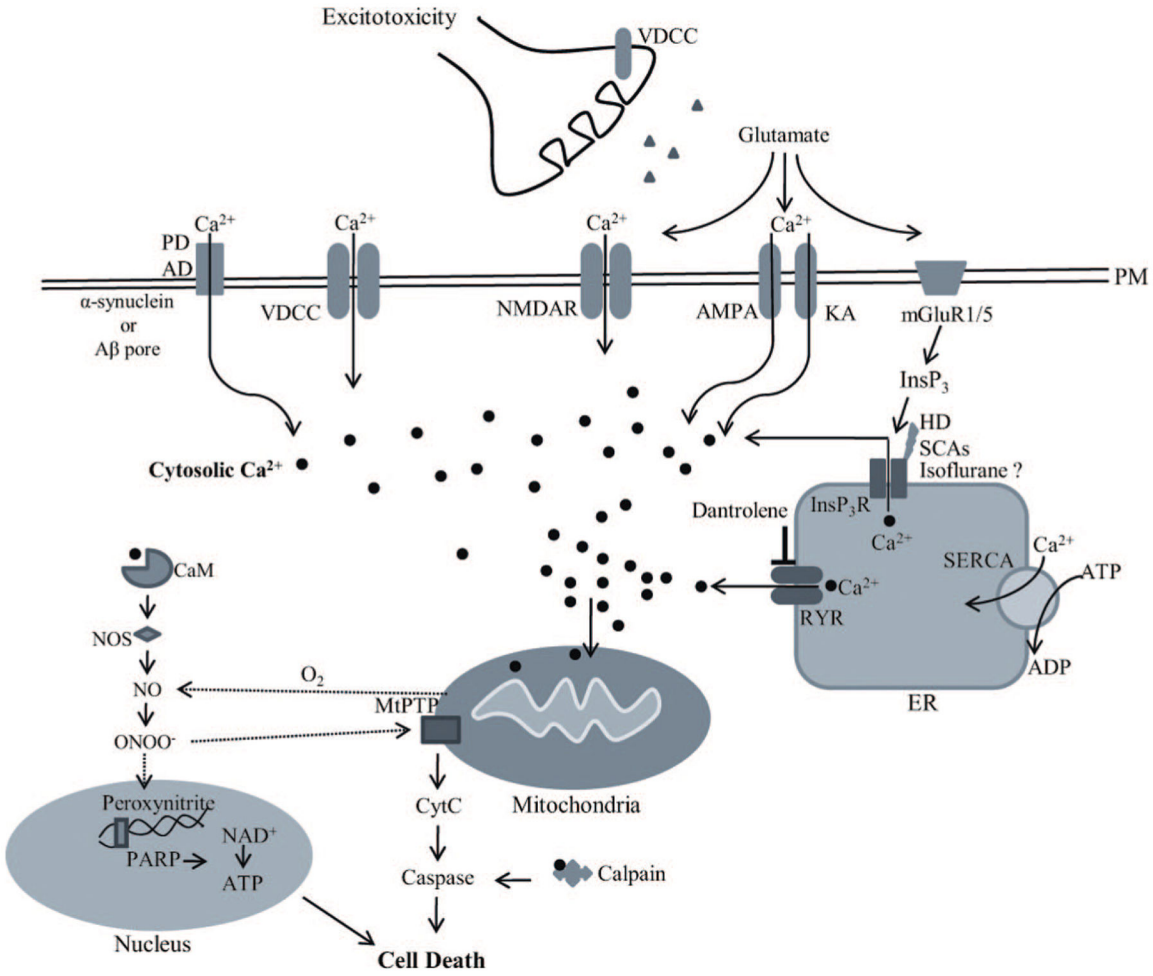

Figure 2.

The models of cytotoxicity induced by Ca^{2+} dysregulation in excitotoxicity (i.e., sepsis, seizure, trauma), neurodegenerative diseases, and general anesthesia. Under normal conditions, $[Ca^{2+}]$ in the extracellular space is 10,000 times higher than the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). Upon electrical or receptor-mediated stimulation, $[Ca^{2+}]_c$ is increased by extracellular Ca^{2+} influx via specific ion channels on the plasma membrane including voltage-dependent calcium channels (VDCCs) and ligand-gated calcium channels or by Ca^{2+} release from intracellular stores. The main intracellular Ca^{2+} store in neurons is the endoplasmic reticulum (ER) and Ca^{2+} is released into the cytosol via activation of ryanodine receptors (RYRs) and inositol-1,4,5-triphosphate receptors ($InsP_3Rs$). Basal $[Ca^{2+}]_c$ is maintained through calcium binding and calcium buffering proteins or uptake into internal stores by the energy-dependent sarco-ER calcium pump (SERCA) at the ER membrane or by the mitochondrial uniporter. Disturbance in Ca^{2+} homeostasis leads to cytotoxicity. Abnormal increase in intracellular $[Ca^{2+}]$ is the result of either increased extracellular Ca^{2+} influx via Ca^{2+} channels or increased release from the ER by either calcium-induced calcium release (CICR) or overactivation of RYRs and $InsP_3Rs$. During excitotoxic conditions (i.e., ischemia, sepsis, seizure, trauma), glutamate release is increased. Glutamate stimulates *N*-methyl-D-aspartate (NMDA), kainate (KA), *α*-

amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and metabotropic glutamate mGluR1/5 receptors. Amyloid-β peptide (Aβ) synthesis is increased in Alzheimer disease (AD) and forms oligomers. Both Aβ oligomers and α-synuclein, which aggregates in Parkinson disease (PD), can form Ca^{2+} permeable pores in the plasma membrane and facilitates the increase of $[Ca^{2+}]_c$. Aβ oligomers also activate NMDA and KA receptors as well as VDCCs and cause Ca^{2+} influx. Activation of mGluR1/5 increases inositol-1,4,5-triphosphate ($InsP_3$), which activates the $InsP_3R$ and causes Ca^{2+} release from the ER. Evidence suggests that the $InsP_3R$ is sensitized to $InsP_3$ during Huntington disease (HD), spinothalamic cerebellar ataxies (SCAs), and general anesthesia by isoflurane. Dantrolene is cytoprotective via inhibiting RYRs and preventing excessive Ca^{2+} release from the ER, especially during CICR. Reduction in ER Ca^{2+} stores results in the misfolding of proteins, which stimulates the unfolding protein response as a cellular stress response. Influx of Ca^{2+} into mitochondria causes the formation of oxygen radicals and energy failure as a consequence of decreased adenosine triphosphate (ATP) production. When cytosolic Ca^{2+} binds to calmodulin (CaM), nitric oxide synthase (NOS) is activated and nitric oxide (NO) is produced. Oxygen radicals react with NO and forms peroxynitrite, which damages DNA and proteins. DNA cleavage activates the DNA-repair enzyme poly (ADP-ribose) polymerase (PARP), which requires energy for its activation. PARP-induced energy depletion worsens cellular stress. Increased $[Ca^{2+}]$ in mitochondria increases mitochondrial permeability via the mitochondrial permeability transition pore (MtPTP), which causes mitochondrial swelling, outer mitochondrial membrane rupture, and release of cytochrome *c* (CytC). CytC activates pro-apoptotic factors (caspases) by reacting with Ca^{2+}-activated calpain and induces apoptosis via intrinsic pathways.