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Effects of COX inhibitors on neurodegeneration and survival in mice exposed to the marine neurotoxin domoic acid

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ABSTRACT

The marine neurotoxin domoic acid (DA) is a rigid analogue of the neurotransmitter glutamate and a potent agonist of kainate subtype glutamate receptors. Persistent activation of these receptor subtypes results in rapid excitotoxicity, calcium-dependent cell death, and neuronal degeneration in regions of the brain where glutamatergic pathways are concentrated. Previous work has shown that DA promotes the expression of inflammatory genes in the brain, such as cyclooxygenase 2 (COX2). To investigate the impact of inflammation on the development of neurodegeneration, and ultimately survival following DA administration, we used selective (L745337, Merck) and non-selective (acetylsalicylic acid (ASA)) COX inhibitors in DA exposed mice. Adult male ICR mice were given a regime of either ASA or L23547 both before and after a single LD50 dose of DA. Mice were observed immediately after toxin introduction and then sacrificed at 2 days post exposure. Our lower dose of L23547 increased survival and was most effective at decreasing neuronal degeneration in the CA1 and CA3 regions of the hippocampus, areas especially sensitive to DA excitotoxicity. This study shows that COX2 plays a role in DA induced neurodegeneration and death, and that inhibitors may be of value for treatment in human and wildlife DA exposure.

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Domoic acid (DA) is a structural analogue of the excitatory neurotransmitter glutamate and has caused disease and death in numerous coastal species including sea birds, sea otters, dolphins, whales and others [18], but is most notable as the basis for routine mass mortality among California sea lions [30]. DA is also the cause of amnesic shellfish poisoning in humans, a term coined due to a characteristic anterograde memory loss [21]. The toxin is produced by several species of diatom within the genus *Pseudo-nitzschia*, a phytoplankton group with worldwide distribution in coastal waters. Bioaccumulation of DA in marine food webs occurs by trophic transfer and poisonings arise most notably via ingestion of contaminated fish, shellfish, and crustaceans. DA induces excitotoxicity by first binding ionotropic glutamate receptors, particularly the kainic acid (KA) and AMPA subtypes [14,28]. This binding is characterized by a persistent, non-desensitizing activation of the receptor, resulting in depolarization, glutamate release, persistent activation of N-methyl-D-aspartic acid (NMDA) receptors, excitotoxicity, and is often accompanied by neurodegeneration [2,24].

The neuropathology in both humans and sea lions is focused on the limbic system of the brain, with lesions most prominent within the hippocampal formation [30,35]. Lesions described range from ischemic neuronal necrosis of the pyramidal cells in cornu ammonis (CA) sectors CA4, CA3 and CA1 in acute cases, with chronic progression to hippocampal sclerosis (or "scarring", with replacement of lost neurons and parenchyma by astrocytes), and hippocampal atrophy. Experimental protocols involving animal models have yielded similar results [29,37]. Regional predilection for limbic system involvement is due to its high concentration of kainic acid and NMDA-type glutamate receptors [9] that are activated by the binding of DA and the subsequent amplification of signals through this closed loop seizure-prone circuitry [25].

Non-steroidal anti-inflammatory drugs (NSAIDs) produce their analgesic, anti-inflammatory, and antipyretic actions via inhibition of cyclooxygenase (COX), which consequently results in decreased synthesis of prostaglandins [38]. To date, two COX isoforms have been identified (COX1 and COX2), along with a splice variant of the COX1 gene often referred to as COX3 [6]. Nonspecific NSAIDS (nsNSAIDs) block both COX1 and COX2 activity, while selective COX2 inhibitors (coxibs) have little effect on COX1. In general, COX1 is thought of as constitutive while COX2 is considered inducible, especially under inflammatory conditions, although COX2 is known to be constitutively expressed in the brain, stomach and kidney

Abbreviations: ASA, acetylsalicylic acid; CA, cornu ammonis; COX, cyclooxygenase; Coxib, COX2 specific inhibitor; DA, domoic acid; iNOS, inducible nitric oxide; NMDA, N-methyl-D-aspartic acid; nNOS, neuronal nitric oxide; NSAIDs, non-steroidal anti-inflammatory drugs.

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[39]. In the brain, COX2 is constitutively expressed only in specific neuronal populations, particularly in the hippocampus [40] and is necessary for synaptic plasticity and memory acquisition [10]. A literature search reveals hundreds of studies touting the neuroprotective value of COX inhibitors in brain injuries and also neurological diseases such as Alzheimers or Parkinsons. However, some results prove contradictory and are difficult to reconcile with the general consensus of its neuroprotective properties under pathologic conditions. As COX2 has been shown via microarrays to be significantly up-regulated in the brains of DA exposed mice [27], and memory formation and learning is clinically impaired following natural and experimental DA exposure [22,31,35], the role of COX in the pathogenesis of DA toxicity warrants further investigation. This study was undertaken to investigate potential benefits of selective and nonselective NSAIDs on survival and neurodegeneration following DA exposure in male ICR mice.

This study was conducted in accordance with NIH guidelines for the ethical care and use of laboratory animals. All animal work was performed by Taconic Biotechnology, Rensselaer, New York and Taconic's animal care and use committee (IACUC) approved the study protocol. One hundred three (103) twelve-week-old male ICR mice (an outbred strain) were supplied by and housed at the Taconic facility. Animals were maintained under virus free barrier conditions with regular health monitoring, kept on a 12:12 h light cycle, and allowed food and water *ad libitum* except for a 1 h period immediately before and after injection with domoic acid. Mice were observed for 1 h following toxin dosing and scored for toxic effects based on the behavioral rating scale used by Tasker et al. to characterize the intensity of DA exposures [33].

Acetylsalicylic acid (ASA, an nsNSAID), better known as aspirin, was obtained from Sigma and resuspended to 28.75 mg/mL in phosphate buffered saline (PBS). An experimental COX2 inhibitor developed and generously donated by Merck, L745337 (5-methanesulfonamido-6-(2,4-difluorothiophenyl)-1-indanone) was resuspended at concentrations of 0.7 mg/mL and 0.14 mg/mL in PBS. The inhibitor L745337 was chosen because it is highly selective for COX2, or in more exact terms, its high ratio of COX2/COX1 inhibition [11]. Domoic acid (DA) was obtained from Calbiochem and resuspended at a concentration of 0.8 mg/mL in PBS.

Mice were divided into 5 treatment groups (20 mice per group) and 1 control group (3 mice). All injections were intraperitoneal (i.p.) and all toxin doses were at the established LD50 of 4 mg/kg [33]. Mice in Group 1 were injected with 100 mg/kg of ASA 1 h before and 8 h after an injection of DA. This dose has shown to be neuroprotective against other neurotoxins [34]. Group 2 mice were injected with 0.5 mg/kg L745337 1 h before and 8 h after DA dosing. Group 3 mice were injected with 2.5 mg/kg L745337 1 h before and 8 h after dosing with DA. Group 4 mice were injected with PBS 1 h prior to dosing with DA, followed by injection with 2.5 mg/kg L745337 8 h after dosing with DA. Group 5 mice were injected with PBS 1 h prior to dosing with DA, and injected with PBS 8 h after dosing with DA. Group 6 control mice received only PBS at T0, and again 8 h later. Repeat dosing of inhibitors was based on a 10 h half life.

Forty eight hours after toxin administration, 31 mice (6 representative of treatment Groups 1–5 and one PBS control mouse) were sacrificed via CO_2 inhalation and intracardially perfused with 10% neutral buffered formalin (NBF). The remaining mice were sacrificed via CO_2 inhalation, decapitated, the calvarium removed and brain exposed, then immersion fixed in NBF for 24 h. Perfusion of six animals per group was performed to hedge against potential artifacts sometimes produced by simple immersion fixation.

Brains were de-identified and sent to a trained pathologist (CAC, DVM, DACVP) for tissue processing and histologic scoring. Approximately 24 h post-euthanasia, the brains were transversely sectioned through the olfactory bulb, septal area, hippocampal area, brainstem, and cerebellum. All sections taken for each brain were placed in a single cassette, routinely processed and embedded in paraffin. Blocks were trimmed and five consecutive slices at 5 μm thickness, containing all the anatomical regions noted, were H&E stained. The Paxinos mouse brain atlas [20] was referenced for neuroanatomical orientation.

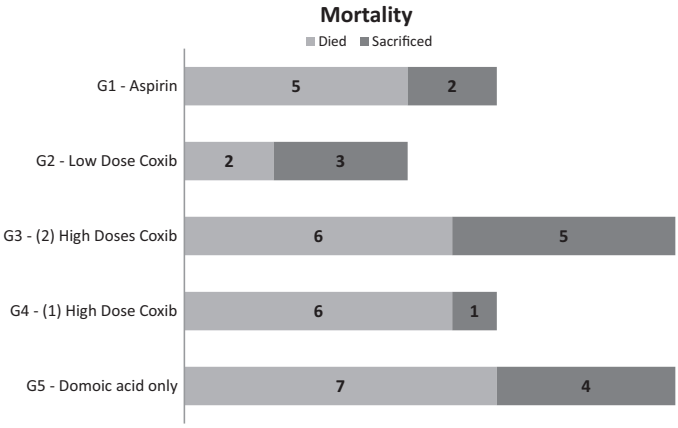

Fig. 1. Mortality. Mice that died as a direct result of toxin exposure are indicated by lighter bar. Mice that were sacrificed and removed from the study due to excessive pain or stress are indicated in darker bar. G indicates group number.

Neurodegeneration was evaluated via light microscopy. The number of degenerative neurons at the level of the dorsal and ventral hippocampus on both the right and left sides, from five consecutive slices, was assessed. Neuronal degeneration was diagnosed if neurons were shrunken with pyknotic nuclei and eosinophilic cytoplasm. The number of degenerative neurons in the dentate gyrus (DG) and the cornu ammonis (CA) segments CA1–4 was assessed for both hemispheres and an average histological damage score was assigned on a 0–3 grading scale for each animal; grade 0, none; grade 0.5, slight (<10%); grade 1.0, mild (10–25%); grade 1.5, mild to selective (25–45%); grade 2.0, selective (46–54%); grade 2.5, selective to extensive (55–75%), and grade 3.0, extensive (>75%), according to a previously defined scale [13]. As the majority of degeneration was limited to the hippocampus, degeneration in other regions was noted when present but not scored.

To assess the effect of each treatment on mortality, a Chi square test for a 2 by 2 contingency table was applied. Mortality in each treatment group (Groups 1–4) was compared to the mortality of Group 5 (toxin only). For neuronal degeneration, a Kruskal–Wallis ANOVA for non-parametric data was used to analyze differences in degeneration between treatment groups for each CA segment, followed by post hoc analysis using Dunn's multiple comparisons to determine which groups were statistically different.

Mice exhibited behavior typical of a high dose, acute exposure to domoic acid (DA). These observations during the first hour post toxin exposure included tonic–clonic seizures, rapid spinning, clawing in the air, stereotypical scratching, and both hyper- and hypoactivity. A small (~20%) reduction in toxicity behavior scoring was noted in Groups 1 (ASA) and 2 (low dose coxib) compared to Group 5 (toxin only) animals. However, this difference was not statistically significant.

Mortality was calculated as the number of mice that were euthanized due to excessive pain and/or distress, in addition to the animals that died directly from the toxic effects of DA (Fig. 1). Chi square analysis to assess if the treatment was statistically significant against mortality between Groups 2 (low dose coxib) and 5 (toxin only) yielded a Chi square statistic of 3.75, well above 2.706 needed for 0.10 probability but just short of the 3.84 needed for the traditional 0.05 probability level to reject the hypothesis that the treatment had no effect. The relative risk of mortality for Group