Cyclooxygenase I and II inhibitors distinctly enhance hippocampal- and cortex-dependent cognitive functions in mice

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Abstract. Cyclooxygenase (COX) enzymes are expressed in the brain; however, their role in hippocampus-dependent and cortex-dependent cognitive functions remains to be fully elucidated. The aim of the present study was to comparatively investigate the effects of piroxicam, a selective COX-I inhibitor, and celecoxib, a selective COX-II inhibitor, on cognitive functions in an AlCl₃-induced neurotoxicity mouse model to understand the specific role of each COX enzyme in the hippocampus and cortex. The AlCl₃ (250 mg/kg) was administered to the mice in drinking water and the drugs were administered in feed for 30 days. Assessments of memory, including a Morris water maze, social behavior and nesting behavior were performed in control and treated mice. The RNA expression of the COX enzymes were analyzed using reverse transcription-quantitative polymerase chain reaction analysis. An ex-vivo 2,2-Diphenyl-1-picrylhydrazyl assay was performed in the hippocampus and cortex. Following 30 days of treatment with the drugs, the mice in the celecoxib- and piroxicam-treated groups exhibited enhanced learning (6.84±0.76 and 9.20±1.08, respectively), compared with the AlCl₃-induced neurotoxicity group (21.14±0.76) on the fifth day of the Morris water maze test. Celecoxib treatment improved social affiliation in the AlCl₃-induced neurotoxicity group, the results of which were superior to piroxicam. Piroxicam led to better improvement in nesting score in the AlCl₃-induced neurotoxicity group. Both drugs decreased the expression levels of COX-I and COX-II in the hippocampus and cortex, and rescued oxidative stress levels. These findings suggested that each drug distinctly affected cognitive functions, highlighting the distinctive roles of COX-I and COX-II in learning and memory.

Introduction

Non steroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenase (COX) enzymes and are used extensively to treat multiple illnesses (1). There are several neurodegenerative disorders, in which concurrent inflammatory stress occurs (1). The importance of neuroinflammation in various neurodegenerative conditions is supported by evidence from post mortem analyses, accompanied by microglial activation and reactive astrocytes (2-4), suggesting the importance of COX enzymes. Several studies have suggested that anti-inflammatory drugs, particularly NSAIDs, appear to be beneficial in slowing the progression of neurodegenerative diseases, including Alzheimer's disease (AD) (5-7), by inhibiting inflammatory responses (8-10). NSAIDs exert their anti-inflammatory effect by inhibiting COX isoforms (11). COX is a homodimer membrane glycoprotein associated with a heme group involved in enzymatic activities (12). Two important isoforms of COX have been identified, COX-I and COX-II (13). Several studies have suggested the beneficial role of COX-II in AD, as the expression and activity of COX-II is increased in early stages of AD, determining the primary protection of NSAIDs in preventing the earlier steps leading to neurodegeneration (14).

There has been controversy regarding the role of COX-I either as a protective or pro-inflammatory agent. COX-I is prominently expressed in microglia (15). Microglial activation is reported following aluminium (Al) administration (16), suggesting the involvement of COX-I following Al-induced injury. COX-I is actively involved in immunoregulation of central nervous system (1,17,18) and its deletion reduces neuro-inflammation and neuronal damage induced by Aβ (19). However, enhanced activity of COX-I is reported as a source of oxidative stress in Aβ-mediated neurotoxicity (13). Multiple studies (19-22) have indicated the active involvement of COX-I in brain injury induced by pro-inflammatory stimuli, including Aβ.

COX-II is expressed in the brain under normal conditions (23), while it is an inducible enzyme in other tissues and is expressed in response to pro-inflammatory stimuli (24). COX-II is prominently expressed in hippocampal and cortical glutamatergic neurons (25), but not in astrocytes and microglial cells (26), suggesting its distinctive role, compared with COX-I. COX-II, which is predominantly present in neurons, is important in regulating brain functions, including synaptic

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plasticity (27,28), however, its specific role in the hippocampus and cortex, which may be involved in cognitive functions, remains to be elucidated. In AD, neuronal levels of COX-II have been found to be elevated either in early stages (15,29,30) or decreased in later stages (31). An association between the induction of COX-II and neuronal degeneration following stimulation of glutamate seizures (32) and spreading of depression waves (33) has also been reported, however, the exact role remains to be elucidated.

Several evidence has supported the protective role of NSAIDs, which inhibit COX-I and COX-II in diseases, including AD, gastric cancer and colorectal cancer (34). Therefore, the balance between COX-I and COX-II may be important to provide balance between the inflammatory response and synaptic plasticity (23). The present study was performed to investigate the distinct role of COX enzymes in hippocampus- and cortex-dependent cognitive function in Al-induced neurotoxicity. Al is a widely used metal and is known as a neurotoxic agent (35). Al causes impaired neurotransmission, oxidative stress (35) and increased lipid peroxidation (36). Studies showed that Al is responsible for the cognitive impairment (37,38). Epidemiologically, there is an association between chronic Al exposure and the incidence of AD (39), and furthermore, elevated levels of Al have been reported in the brains of AD patients (40).

To understand the role of COX enzyme inhibition in cognitive function, the present study administered mice with piroxicam and celecoxib at specific doses to inhibit the COX enzymes and to examine their contribution in hippocampal- and cortex-dependent cognitive functions. This investigation aimed to determine the distinct roles of COX-I and COX-II and examine the effects of celecoxib and piroxicam on organizational behavior, sociability, depression, anxiety and oxidative stress, which is a hallmark of AlCl3-induced neurotoxicity.

Materials and methods

Drugs and chemicals. Aluminium Chloride hexa hydrate (cat. no AL0770) was purchased from Scharlab (Barcelona, Spain). Celecoxib 100 mg capsules (cat. no. 064C01) and piroxicam 20 mg capsules (cat. no. 12C018) were purchased from Getz Pharma Private Limited (Karachi, Pakistan) and Global Pharmaceuticals (Chalfont, PA, USA), respectively. 2,2-Diphenyl-1-picrylhydrazyl (DPPH; cat. no. 101087701) and diethylether (cat. no. 676845) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals. Male Balb/c mice weighing 35-45 g were provided by Amson Vaccines and Pharma, Ltd. (Islamabad, Pakistan). All experiments performed complied with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (1996) (41) and the protocol was approved by the ethical committee for research on animals (Internal Review Board, Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad, Pakistan). The animals were maintained in the animal house (three mice/cage) at Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, under controlled conditions (23-25°C; 10 h light/dark cycle), and house separately according to the group to which they pertain. The experimental mice were provided with access to distilled water and a standard diet ad libitum.

Drug administration. In the present study a previously reported mouse model was used (42) with certain modifications. A total of four groups of animals were included, in which treatment was performed in to the respective groups for a duration of 30 days; I) Control group, 10 animals were provided with distilled water and a standard diet; II) AlCl3-induced neurotoxicity group: 10 animals were administered with AlCl3 (250 mg/kg/day) dissolved in distilled water; III) Celecoxib-treated group (AlCl3+Cel), 10 animals were provided with AlCl3 (250 mg/kg/day) dissolved in distilled water, and celecoxib was provided in the feed at the dose of 15.6 mg/kg body weight per day; IV) piroxicam-treated group (AlCl3+Pxm), 10 animals were provided with AlCl3 (250 mg/kg/day) dissolved in distilled water and piroxicam was provided in the feed at a dose of 12.5 mg/kg body weight per day. The administration doses for the Al and the drugs were calculated based on the water and diet consumption of the animals prior to initiation of the experiments in the present study. None of treatment approaches affected the water or food intake of the mice, or affected weight changes in the groups of mice (data not shown).

Behavioral assessment

Morris water maze test for assessment of spatial memory.

The procedure for assessing spatial reference memory was the same as that described previously (43) with modifications. On the 25th day of treatment, the animals were subjected to a Morris water maze test, which continued until the end of the experiment. The experimental apparatus used was comprised of a circular water tank filled with water, with an invisible platform placed below the surface of the water. The temperature of the water was 21-23°C, and the water was placed in an assessment room and clues external to the maze were visible from pool for spatial orientation by mice. These clues were maintained constant throughout the task. The pool was divided into four equal quadrants. During spatial reference memory training, the platform was always placed in the same spatial location of the pool and the releasing positions of the mice were changed in every trial. The mice received five trials per day for consecutive five days. Each trial duration was 60 sec, with an inter trial interval of 10 mins. The time taken by the mouse to reach the platform was recorded.

Social preference test. The assessment of social preference was performed as described previously (44). Two sessions of 10 min were performed, with 20 min gap between them. In the first session, the test animal was exposed to a mouse, which was confined to a small closed cage, while the second cage in the testing box was empty. The mouse was allowed to interact with the mouse and an empty cage. Following the first session, the animal was returned back to its housing cage for 20 min. During the second session, the stranger mouse was placed in the empty cage and the test mouse was allowed to interact and the time of interaction was recorded. The social novel preference was recorded and the discrimination index (DI) for the two sessions was calculated; which is the ratio between the time spent with mouse A (session I) or stranger mouse (session...
II) and the total interaction time, according to the following equation: $DI = \frac{\text{time spent with mouse A or mouse B}}{\text{total time of interaction}}$.

**Nesting behavior.** Nesting behavior was assessed, as described earlier (45) and the nest was scored from 0-5. Score 1, 90% cotton was untouched by mouse; score 2, 50-90% of cotton was torn up; score 3, mostly shredded cotton.; score 4, completely shredded cotton only with one or 2 walls. Score 5, walls higher than mouse body height with perfect nest. Assessment was performed in individual cages, normal bedding was used and each cage was provided with 4 g of cotton for making a nest. The mice were placed in these cages with cotton provided overnight, and the results were assessed the following day.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for RNA expression analysis.** The protocol was adopted as explained earlier (46) to examine the effect of COX inhibitors on gene expression following treatment with respective drugs. The animals were sacrificed by decapitation under diethylether anesthesia, and their brains (50-100 mg; four samples/group) were isolated to extract the hippocampus and cortex. TRIzol was used to extract total RNA. The quality of the RNA was assessed by running on agarose gel to obtain two ribosomal RNA bands, and the quantity was determined using a spectrophotometer (Optima SP300; Optima Inc., Tokyo, Japan). Equal quantities of RNA were used (1 µg RNA in 40 µl of reaction mixture) for RT into cDNA. cDNA (3 µl) was used for the PCR reactions with at total reaction mixture (10 µM) containing MgCl2 (25 µM), dNTPs (10 µM) and Taq polymerase (0.625 U/25 µl) (Thermo Fisher Scientific, Inc.). The PCR thermocycling (2720 Thermal Cycler; Applied Biosystems Life Technologies, Foster City, CA, USA) was performed with the following conditions: Initial denaturation for 95˚C for 5 min, followed by denaturation at 94˚C for 30 s, annealing (temperatures indicated in Table I) for 30 s, and extension at 72˚C for 30 s with the indicated number of cycles. This was followed by a final extension step at 72˚C for 10 min. Separation of the amplified PCR products was performed on a 2% agarose gel (Merck Millipore, Karachi, Pakistan) with ethidium bromide (Sigma-Aldrich) for staining. The quantification of each PCR product band was determined using Image J 1.47 software (National Institutes of Health, Bethesda, MD, USA). Actin was used as a housekeeping gene to normalize the respective group of PCR products.

**Figure 1. Morris water maze.** (A) Comparison of learning and memory in the control, AlCl3-treated group and drug-treated groups. (B) 5th day trial, the average of the trial was plotted and the bar diagram shows the effect of the respective drugs on the escape latency. $^{**}P<0.01$, compared with the AlCl3-treated group. Error bars represent the mean ± standard error of the mean (n=10; analysis of variance, followed by Bonferroni’s comparison test). Cel, celecoxib; Pxm, piroxicam.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Annealing temp (˚C)</th>
<th>Cycles (n)</th>
</tr>
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| Actin  | Forward: GCCTTCCTTCTGGGTATGG  
Reverse: CAGCTCAGTAACAGTCCGC | 55                  | 32         |
| COX-1  | Forward: CTACATCGCTGGAGGATCCT  
Reverse: CGTCAGCAGTGGTGACTTA | 55                  | 35         |
| COX-2  | Forward: CAGGTCTATTGGTGAGGAGG  
Reverse: CATGTTCCAGGAGGATGAGG | 54                  | 35         |

COX, cyclooxygenase.

Assessment of ex-vivo antioxidant activity using a DPPH radical scavenging assay. The antioxidant activity in brain samples were evaluated using a DPPH (Sigma-Aldrich) radical scavenging assay, as described earlier (47) with certain modifications. The control, AlCl3-treated, celecoxib-treated and piroxicam-treated brain samples, with a 0.1 mg/ml protein concentration, were homogenized in 1 ml methanol. Subsequently, 0.4 ml of 0.1 mM DPPH was added to the homogenized brain tissue samples which were designated as test samples. Pure DPPH solution was used as a control. The solutions were incubated at 37˚C for 30 min and the absorbance was measured at 517 nm using an Optima SP300 spectrophotometer. The percentage DPPH inhibition was calculated by using the following formula, and was normalized to per/mg protein: DPHH inhibition (%) = (absorbance of control - absorbance of test sample / absorbance of control) x 100.
Statistical analysis. Data are expressed as the mean ± standard error of the mean and the results were statistically analyzed using GraphPad Prism software. One way analysis of variance was used followed by Bonferroni’s comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of celecoxib and piroxicam on learning and memory. The control, AlCl₃-treated, celecoxib and piroxicam treatment groups were investigated in a spatial reference memory task using a Morris water test (Fig. 1A). The result of the trial on the fifth day demonstrated a significant (P<0.01) improvement of memory in the celecoxib-treated mice (6.84±0.76 sec) and piroxicam-treated mice (9.20±1.08 sec), compared with the AlCl₃-treated mice (21.14±0.76 sec; Fig. 1B).

Effect of celecoxib and piroxicam on social behavior. Social affiliation and social novelty preference assessments were performed to examine the effect on sociability and preferences for social novelty. The comparison revealed that, in session I, the mice in the AlCl₃-treated group spent less time (28.8±8.97 sec) with the familiar mouse (mouse A), compared with the control group (73.9±10.97 sec), however, the mice in the control group spent less time in the empty cage (Fig. 2A). During session I, the mice in the celecoxib and piroxicam treatment groups exhibited elevated social interaction, spending a longer duration with mouse A (104.5±12.29 sec and 70.90±12.84 sec, respectively; Fig. 2A).

In session II, the time spent with the stranger mouse (mouse B), compared with mouse A was calculated. The control group spent significantly (P<0.001) more time (57.5±6.18 sec) with mouse B, compared with mouse A, compared with the AlCl₃-treated group (23.2±3.31 sec), which demonstrated lack of social novelty preference (Fig. 2B). The celecoxib (60.5±7.52 sec) and piroxicam treatment groups (87.80±13.89 sec) exhibited a significant social novelty preference (Fig. 2B).

In determining the DI of the mice in session I, piroxicam (0.7±0.02) exhibited significantly better effects than the celecoxib group (0.75±0.03) when the two drug treatment groups were compared with the AlCl₃-treated group (0.50±0.5; Fig. 2C).

The DI calculated of the mice in session II indicated that the control group demonstrated better social novelty preference (0.83±0.06), compared with the AlCl₃-treated group (0.50±0.5; Fig. 2C).

Effect of celecoxib and piroxicam on nesting behavior. Nesting behavior was assessed to determine the organizational and daily activities of living in mice. As shown in Fig. 3, the nest score of the AlCl₃-treated group (2.9±0.23) declined, compared with the control group (4.6±0.22). Piroxicam was effective and improved nesting score (4.2±0.2), whereas celecoxib (3.10±0.43) was not effective (Fig. 3).

Effect of celecoxib and piroxicam on gene expression. In the present study, RT-qPCR analysis was performed to examine the effect of drug treatment on gene expression. In the hippocampus, there was an increase in the levels of COX-I (2.8±0.34) in the AlCl₃-treated group, compared with the control group (0.74±0.15), however, only piroxicam treatment decreased the expression of COX-I significantly (0.9±0.32), whereas, celecoxib was not effective (Fig. 4A).
In the cortex, a significant (P<0.01) increase in the level of COX-I (1.57±0.16) was observed in the AlCl₃-treated group, compared with the control group (0.8±0.03). Piroxicam treatment resulted in significant (P<0.05) downregulation in the expression of COX-I (0.9±0.06), compared with the AlCl₃-treated group. Celecoxib treatment (1.24±0.2) was not found to be effective (Fig. 4B).

In the hippocampus, upregulation in the levels of COX-II (2.65±0.43) were observed in the AlCl₃-treated group, compared with the control group (0.7±0.09; Fig. 4C), exhibiting inflammatory stress. Celecoxib treatment resulted in significant (P<0.01) downregulation in the levels of COX-II (1±0.24), indicating its selective effect on the gene expression of COX-II, whereas piroxicam treatment was not effective (1.60±0.23; Fig. 4C).

In the cortex, the levels of COX-II were elevated in the AlCl₃-treated group (2.4±0.30), compared with the control group (0.90±0.20). The celecoxib and piroxicam treatment groups exhibited downregulated levels of COX-II (0.90±0.13 and 1.46±0.06, respectively), compared with the AlCl₃-treated group (Fig. 4D).

**Ex-vivo DPPH assay.** To investigate the effect of celecoxib and Piroxicam on oxidative stress, a DPPH assay was performed in the hippocampus and cortex of the brain tissues of the mice in the treatment groups. The results demonstrated that the AlCl₃-treated group exhibited a substantial load of free radicals and a decreased percentage of DPPH inhibition (14±2.7%) in the hippocampus, also indicative of decreased endogenous anti-oxidants, compared with the control (44.6±1.07%; Fig. 5A). The celecoxib-treated group (28.2±1.8%) exhibited a significant (P<0.001) increase in the percentage inhibition of free radicals in the hippocampus, whereas the piroxicam-treated group was less effective (24±1.51%; P<0.01; Fig. 5A).

In the cortex, the AlCl₃-treated group (27.51±14.87%) exhibited increased oxidative stress resulting in free radical production, compared with the control group (71.54±4.85%; Fig. 5B). Celecoxib treatment led to the effective inhibition of the free radicals (72.4±5.4) induced by AlCl₃. Similarly, piroxicam treatment led to increased free radical scavenging activity (65.2±6.02%), compared with the AlCl₃-treated group (Fig. 5B).

**Discussion**

The present study attempted to identify which COX enzyme inhibition is predominantly responsible for the improvement in hippocampal- and cortex-dependent cognitive function in the AlCl₃-treated mice model to determine the role of NSAIDs in neurodegenerative disorders. The present study demonstrated the significant effect of celecoxib and piroxicam on learning and memory, determined using the Morris water maze test. The
two drugs exhibited similar efficacy in the Morris water maze, which is a hippocampus-dependent memory task. These results are concordance with those of earlier studies, which reported that selective COX-II inhibition restores memory function in APP-overexpressing transgenic mice (48) and selective COX-I inhibition promotes learning (11). Treatment with celecoxib and piroxicam demonstrated memory enhancing effects by decreasing the expression levels of the COX-I and II isoforms in mice, suggesting that decreased expression levels may have decreased inflammation and increased memory. Another possible reason for the enhanced memory in the COX-II inhibitor-treated group is the inhibition of overexpressed COX-II in the hippocampus, resulting in improved memory. COX-I inhibition may improve memory through decreasing inflammation, however, the exact mechanism remains to be elucidated.

Social affiliation and social novelty preference are amygdala- and cortex-dependent behaviors (49). The present study suggested that celecoxib exhibited improved effects on social affiliation (session I), whereas piroxicam exhibited a more marked effect on social novel preference. It has been revealed that COX-II inhibition is beneficial in suppressing the stress induced by elevated COX-II enzyme in rat brain (50). Similarly, the role of piroxicam in novel social preference is a novel finding. The present study investigated, for the first time, the effect of the two COX inhibitors in an AlCl₃-induced neurotoxicity mouse model, and demonstrated that COX inhibitors assist in improving social recognition memory, suggesting their potential role in neurodegenerative conditions accompanied with social memory problems. Further investigations are required to determine the importance of the effect, and to investigate the mechanism through which they act to improve these symptoms in neurodegeneration.

Nest building is a common behavior in mice and is associated with the maintenance of body temperature (51). It is a prefrontal cortex- and hippocampus-based behavior (52), and it has been reported that damage in the medial prefrontal cortex and hippocampus leads to the reduction in nesting material consumption and disturbs the quality of the nest (52,53). The present study revealed that piroxicam improved the quality of the nest and reversed Al-induced impairment, whereas, celecoxib failed to produce a significant effect. These are novel findings and suggest an additional pharmacological role of piroxicam, however, the exact underlying mechanism remains to be elucidated.

In the present study, the levels of COX-I and COX-II were elevated in the hippocampus and cortex in the AlCl₃-treated group, and piroxicam reduced the expression levels of COX-I in hippocampus and cortex, which may be its underlying mechanism in improving cognitive functions. This drug has not been investigated previously for its effect on gene expression in the AlCl₃-treated mouse model. Other COX-I inhibitors have been investigated and have offered protection against mild to moderate cognitive impairment in patients with neurodegenerative disease (54). Celecoxib treatment also led to reduced expression levels of COX-II in the hippocampus and cortex, suggesting its beneficial role in reducing neuroinflammation, which differs to earlier reports that selective COX-II inhibitors fail to demonstrate beneficial effects in patients with neurodegenerative disease (7,55). Therefore, these findings suggested that depressive symptoms of disease may be treated using celecoxib.

It has already been accepted and established that oxidative stress is one of the hallmarks of several neurological disorders, particularly AD (56). In the present study the AlCl₃-treated model exhibited increased oxidative stress in the brain tissue, compared with the control, which was concordant with an earlier study, confirming the role of Al in producing oxidative damage in brain tissues (57). The ex-vivo anti-oxidant activity of piroxicam and celecoxib exhibited increased free radical inhibition in the hippocampus, compared with the AlCl₃-treated group. In the cortex, the two drugs equally decreased oxidative stress, indicating their therapeutic potential in neurodegenerative disorders.

In the present study, comparison of piroxicam and celecoxib in reference to Al-induced neurodegeneration was performed for the first time. The ability of piroxicam to improve organizational behavior and sociability are significant findings, suggesting the role of piroxicam in various neurodegenerative disorders. Celecoxib treatment markedly improved cognitive functions, including learning, memory and anxious behavior. Its effect on social activity was also examined, which exhibited positive effects as a novel finding. The two drugs also improved AlCl₃-induced neuroinflammation and decreased oxidative stress, which demonstrates their potential for use in neurodegenerative diseases. These results suggested that COX enzymes are important in neuropathology and have potential as drug targets in neurodegeneration. This investigation can be broadened to further investigate the possible molecular mechanisms of these drugs in other neurodegenerative conditions.

Figure 5. Percentage DPPH inhibition/mg of protein in the (A) hippocampus and (B) cortex. Comparison between the control, AlCl₃, AlCl₃+Cel and AlCl₃+Pxm treatment groups are shown in 100 µg/ml brain tissue samples. Error bars represent the mean ± standard error of the mean. *P<0.05 and **P<0.01, compared with the AlCl₃-treated group (analysis of variance followed by Bonferroni's comparison test). DPPH, 2,2-Diphenyl-1-picrylhydrazyl; Cel, celecoxib; Pxm, piroxicam.
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