Diclofenac Sodium Inhibits Hepatic Tryptophan 2,3-Dioxygenase but Augments Brain Indoleamine 2,3-Dioxygenase Activities in Rats

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Abstract: Tryptophan 2,3-dioxygenase (TDO) exist only in liver while indoleamine 2, 3-dioxygenase (IDO) exists ubiquitously in the body, these are the most rate-limiting enzymes of kynurenine pathway (KP). In response to elevated levels of cortisol and pro-inflammatory cytokines, both enzymes show increase activity in patients with depression or Alzheimer disease (AD). Non-steroidal anti-inflammatory drugs may protect against both depression and AD, but observational studies have offered contradictory results. Present study evaluates the effects of anti-inflammatory diclofenac sodium (DS) on rat hepatic TDO and brain IDO activities. Adult Albino Wistar rats were divided into control and test groups, each test group received DS (2mg/kg) i.p. injection daily and were killed either after 3.5 hours (acute treatment) or after 3, 5 and 7 days (chronic treatment) while control groups received an equal volume of vehicle. Results show that TDO enzyme activity was inhibited and liver tryptophan concentrations were increased after 3 to 7 days treatment of DS; however no effect was seen on these parameters after 3.5hrs. Brain IDO activity was increased after both acute and chronic DS treatment. It is concluded that DS inhibits hepatic TDO enzyme activity following chronic treatment, while augments brain IDO activity following both acute and chronic DS treatment, this may result in rise in cerebral kynurenic acid and/or quinolinic acid concentrations. Therefore there is a need that effects of DS on kynurenine pathway should be further investigated to rule out the protective effect of DS in inflammation-induced depression and Alzheimer disease.

Keywords: Dicolfenac sodium, tryptophan, kynurenine, indoleamine 2,3-dioxygenase, tryptophan 2,3-dioxygenase.

INTRODUCTION

Indoleamine 2, 3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) are regulatory enzymes of kynurenine pathway (KP). The induction of IDO and TDO leads to increased concentration of kynurenine pathway metabolites, mostly kynurenic acid and quinolinic acid. Recent studies confirmed that depression can originate from changes in the tryptophan availability following TDO activation, and that similar alteration in KP activity is observed in Alzheimer disease (AD) patients. In response to elevated levels of cortisol and pro-inflammatory cytokines, both enzymes show increased activity in patients with depression or dementia [1]. The hyperactivity of the enzymes subsequently increases the catabolism of tryptophan via the KP. Inflammation was shown to be a key factor in depression as well as AD, and multiple of the involved inflammatory factors show comparable changes in both diseases. Similarly, the severity of depressive symptoms in patients with major depression was correlated with activity of IDO, the rate-limiting enzyme of the KP [2]. KP is upregulated during infection and is activated by proinflammatory cytokines [3] and is shown to be involved in neuro-immunological disorders including Alzheimer's disease [4] and major depression [5]. Decreases in some of the monoamines are found in both depression and dementia. The monoamine which is most intensively studied in depression research is 5-hydroxytryptamine (5-HT; serotonin) a neurotransmitter synthesized from the essential amino acid tryptophan. An increase in serotonin turnover, resulting in decreased levels of serotonin is found in human depressed patients and in mice models [6, 7]. Pharmaceutical treatment with SSRIs, which functions through normalizing serotonin levels, is a common treatment method for depression [8] 5-HT synthesis depends mainly on the availability of tryptophan precursors in the brain (and it has a competitive relation with amino acids such as valine, leucine and phenylalanine for the same cerebral transport mechanisms at the level of the blood-brain barrier). Cytokines reduce tryptophan availability by activating the enzyme which metabolizes it, indoleamine 2,3-dioxygenase. Such an excessive indoleamine 2, 3-dioxygenase stimulation can lead to a tryptophan decrease in serum, which is accompanied by a significant reduction of 5-HT synthesis. Low tryptophan availability in the brain can become the main event underlying the deficit of serotonin, which accompanies depressive disorder [9].

Since a tryptophan depletion study in humans indeed observed a linear relationship between

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decreased tryptophan levels and the severity of depressive symptoms, IDO activation was hypothesized to be involved in the transition from cytokine-induced sickness to major depression [10]. The hyperactivity of the enzymes subsequently increase the catabolism of tryptophan via the KP, which results in the production of neuro-active metabolites, of which quinolinic acid (QUIN) had the most detrimental effects [11]. Increased levels of QUIN are reported to lead to dysfunction of neurons and even neuronal death, thereby inducing permanent damage. Diclofenac sodium (DS) a non-steroidal anti-inflammatory drug (NSAID) is a prescription medicine used to relieve pain and swelling. NSAIDs play role in modulating IFN-alpha-induced neurochemical alterations, and raise the possibility of the use of NSAIDs for the prevention of IFN-alpha-induced depression [12]. NASAIDs have anti-amyloidogenic effects for Alzheimer's ß-amyloid fibrils *in vitro* [13]. The present study helps us to gain an insight on effects of DS administered on TDO and IDO activities.

MATERIALS AND METHODS

Animals and Treatment

All animal procedures described below were conducted in strict accordance with the national research council for the care and use of laboratory animals (1996). Ethical approval was obtained from institutional animal ethics committee, University of Karachi. All efforts were made to minimize the number of animals and any pain/distress they might incur. Locally bred male Albino-Wistar rats weighing 150-250 grams were used in the study. Animals were housed in plastic cages. Each group has six animals, for the control and test groups. The animals were kept under natural 12hr light-dark cycle at room temperature (23±2 ºC). The animals had free access of standard lab chow and clean water and were acclimatized to the new environment before any treatment. Control group received Saline (0.9% NaCl) administered intraperitoneally according to body weight. Rats were killed by decapitation using a guillotine. The test animals were grouped as acute treatment (killed after 3.5 hrs) and chronic treatment (killed after 3,5 and 7 days). DS (2mg/kg/3ml) was injected intraperitoneally daily and were killed by decapitation using guillotine at respective time intervals assigned for the groups. Livers were perfused and whole brains were dissected out. The liver and brain samples were stored at -70°C until analysis.

Biochemical Determinations

Tryptophan 2,3-dioxygenase activity was determined in rat liver homogenates (2gm of perfused frozen liver tissue in 13ml of 0.14M KCl (pH 7.0) was homogenized at 0ºC using Polytron homogenizer spinning at 13000 rpm for 2-3 minutes), either in absence (holoenzyme activity) or in the presence (total enzyme activity) of added haematin 2M (haematin dissolved in 0.1M NaOH) as previously described in detail [14]. The apoenzyme activity was quantified as the difference between total and holoenzyme activities.

Preparation of Brain/ Liver Extract

In brief, whole brain or liver (1gm) tissue were homogenized in 1 ml ice cold water and 2 ml $HClO_4$. Mixture was homogenized for 1 minute using an ultra turrax homogenizer; the homogenates were allowed to stand in ice-cold tubes for 10 minute before centrifugation at 6000 rpm for 10 min at 4^0C. The supernatants were decanted and adjusted to 4 ml with 6% (w/v) $HClO_4$; 0.5 ml portion of each homogenate was filtered A 0.5 ml portion of each homogenate was filtered and used or stored [15].

A simple, rapid isocratic liquid chromatographic procedure with ultraviolet and fluorimetric detection was adapted for the separation and quantification of L-tryptophan and kynurenine in either liver / brain extracts [15]. Tryptophan was monitored by means of its native fluorescence at the excitation wave length of 254 nm and emission wave length of 404 nm whereas kynurenine was detected simultaneously by UV absorption at the wave length of 220 nm. The brain IDO activity was estimated by calculating the serum Kynurenine-to-Tryptophan ratio (Kyn/Trp ratio).

Drug Preparation

2 mg DS was dissolved in vehicle ethanol: saline solution (1:2). Drug was administered intraperitonially according to the body weight of rats.

Chemicals and Drugs

L-tryptophan and Kynurenine from Sigma chemical Co (St, Louis, Mo), Diclofenac Sodium was gift from Indus Pharma (Pvt.) Ltd. All others chemicals used were of highest analytical grade.

Statistical Analysis

The results were expressed as mean ± SEM. The data were analyzed by one-way ANOVA followed by

post hoc Turkey's test. p<0.05 was considered as statistically significant (SPSS version 15.00).

RESULTS

Table **1** shows acute and chronic effects of DS (2 mg/kg/3ml) on hepatic TDO enzyme activity. Data analyzed using One-way ANOVA shows a significant effect of drug (F=300; p<0.01, F=637; p<0.01, F=40.42; p<0.01) on holo, total and apo enzyme activity respectively. Individual comparison by Tukey's test shows that there were significant decrease in holo enzyme activity by 35% (p<0.01), 71%, (p<0.01) after 3 and 5 days respectively. There was a non-significant change observed in holoenzyme activity after 3.5hrs (acute) treatment in contrast there was significant decrease by 32% (p<0.01) after 7 days treatment. Significant decreases in total enzyme activity by 46% (p<0.01), 70% (p<0.01) and 47% (p<0.01) were observed after 3, 5 and 7 days treatment respectively. There was a significant decrease in apo enzyme activity by 62% (p<0.01) 74% (p<0.01) and 69% (p<0.01) after 3, 5 and 7 days treatment respectively. Table **1** also shows acute and chronic effects of DS (2 mg/kg/3ml) administration on rat liver TRP concentrations. Data analyzed using One-way ANOVA shows a significant effect of drug (F=165; p<0.01). Individual comparison by Tukey's test showed significant (p<0.01) increase by 104% -107%in 3 -7 days treatment respectively.

Table **2** shows effects of DS (2mg/kg/3ml) on brain tryptophan, kynurenine and IDO activity (Kynrunine/tryptophan ratio). Data analyzed by one-way ANOVA shows significant effect of drug (F=249; p<0.01, F=43; p<0.01 and F=22.3; p<0.01) on brain TRP, KYN and KYN/TRP ratio respectively. Individual comparison by Tukey's test shows significant decrease in brain TRP (27%, p<0.01) (9.6%, p<0.01) after 3.5hrs and 3-days treatment in rats. In contrast brain TRP levels were significantly increased by 25% (p<0.01) and 42% (p<0.01) after 5 and 7 days treatment respectively. Brain kynurenine concentrations were significantly increased by 55 % (p<0.05), 65% (p<0.01), 190% (p<0.01) and 216% (p<0.01) after 3.5hrs, 3, 5 and 7 days treatments respectively. Table **2** also shows significant increase in IDO activity by 125% (p<0.01), 79%, (p<0.01), 129%(p<0.01) and 116% (p<0.01) after 3.5hrs, 3, 5 and 7 days treatments respectively.

DISCUSSION

TDO (Tryptophan 2,3-dioxygenase; EC 1.13.11.11) is a cytosolic haem dioxygenase that catalyzes the oxidative cleavage of the C_2-C_3 bond of the indole ring of L-Tryptophan. This reaction is the first rate-limiting step of the kynurenine pathway of tryptophan

Table 1: Effects of Diclofenac Sodium Administration on Rat Liver TDO Enzyme Activity

Parameter	Diclofenac Sodium Treatment					One –way ANOVA
Tryptophan 2,3 dioxygenase activity (µM of Kynurenine formed/h/g wet.wt. of liver	Control	0 day (3.5 hrs)	3 days	5 days	7 days	Df (1,25)
Holo enzyme	2.78±0.24	3.2±0.06	1.78±0.06*	0.8±0.04*	1.9±0.11*	F=300 P<0.01
Percentage Change			-35%	-71%	-32%	
Total enzyme	4.7±0.2	4.8±0.02	2.5±0.1*	1.4±0.02*	2.5±0.03*	F=637.9 P<0.01
Percentage change			-46%	-70%	-47%	
Apo enzyme	1.95±0.16	1.58±0.08	0.73±0.05*	0.5±0.05*	0.6±0.12*	F=40.42 P<0.01
Percentage change			-62%	-74%	-69%	
Liver TRP (µg/g wet. wt. of liver)	9.95±0.09	9.45±0.09	20.3±0.05*	20.5±0.05*	20.6±0.06*	F=165 P<0.01
Percentage Change			+104%	+106%	+107%	

Experimental details are given in the materials and methods section. All values are mean ± SEM of six rats in each group. Treated group was administered Diclofenac Sodium (2mg/kg/3ml) (i.p) daily and were killed either at 3.5 hours or after 3, 5 and 7 days. Control animals received an equal volume of vehicle. Data was analyzed using one-way ANOVA followed by post-hoc Tukey's test. Significant differences between the drug treated groups and the control are indicated by * p<0.01.

Table 2: Effects of Diclofenac Sodium on Brain TRP, KYN and IDO Activity in Rats

Parameter	Saline Control	Diclofenac Sodium Treatment				One-way ANOVA
		3.5 hrs (0 day)	3 days	5 days	7 days	Df (1,25)
Brain TRP (µg/g wet wt. of tissue)	3.3±0.06	2.4±0.08*	2.98±0.02*	4.15±0.02*	4.7±0.06*	F=249.0 P<0.01
Percentage change		-27%	-9.6%	+25%	+25%	
Brain KYN (ng/g wet wt. of tissue)	80±5.6	124±4.4	132±8.1*	232±8.2*	253±9.5*	F=43.4 P<0.01
Percentage change			+65%	+190%	-16%	
IDO activity (KYN/TRP ratio)	24.2±1.16	54±4.0*	43.4±2.8*	55.5±9.5*	52.5±6.7*	F=22.3 P<0.01
Percentage change		+125%	+79%	+129%	+116%	

Experimental details are given in the materials and methods section. All values are mean ± SEM of six rats in each group. Diclofenac Sodium (DS) was administered i.p. (2mg/kg/3ml) daily and were killed either at 3.5 hours or after 3, 5 and 7 days. Control animals received equal volume of vehicle. Data was analyzed using one-way ANOVA followed by Tukey's post-hoc analysis. Significant differences between the drug treated groups and the control are indicated by *p<0.01. TRP= tryptophan, KYN=kynurenine. IDO = indoleamine 2, 3-dioxygenase.

catabolism, which finally leads to the formation of nicotinamide dinucleotide (NAD^+), a process considered as the principal biological function of TDO [16]. TRP accessibility to the brain also shows an important role in central 5-HT synthesis because tryptophan hydroxylase, the rate limiting enzyme of the serotonin biosynthetic pathway, is unsaturated with its substrate (TRP). Brain TRP concentration is the most important single metabolic factor of the rate of serotonin synthesis. Subsequently, peripheral factors influencing circulating TRP availability to the brain play important roles in serotonin synthesis. These include, at the crucial level of control, activity of the major TRP degrading enzyme, liver TDO [17]. The present results have shown that acute treatment with DS has no significant effect on hepatic TDO activity. However, chronic administration of DS has got an inhibitory effect on TDO activity. In this case, inhibition of TDO was due to the inhibition of conjugation of the apoenzyme with haem prosthetic group or due to the defective synthesis of the apoenzyme. These observations are in accordance with findings reported earlier by different antidepressants like imipramine, paroxetine, fluoxetine, citalopram, moclobamide, tianeptine, Saint John Wort and NSAIDs like salicylate [18, 19, 20] that share a common property of inhibiting TDO activity thereby increasing brain TRP. The TDO enzyme activity is inversely proportional to the brain 5-HT concentration and/or turnover has been documented under many circumstances [21, 22, 23]. TDO regulates serotonin synthesis by different mechanisms such as conditional availability of circulating free TRP (non-protein bound) to the brain, either the TDO activity increased or decreased, direct inhibition of cerebral uptake of TRP by metabolites, especially levels of KYN that are increased following TDO activation [24]. Decrease availability of pyridoxal phosphate, for the activity of 5-hydroxytryptophan decarboxylase in brain due to increased hepatic demand for coenzyme in reactions along the kynurenine pathway after TDO enhancement [25]. Pro inflammatory cytokine stimulate the activity of IDO which deplete TRP levels hence increasing the level of neurotoxic metabolites of the kynurenine pathway, these two hypotheses is related with depression (TRP depletion and kynurenine toxicity). Treatment with antidepressants may correct imbalances in these inflammatory markers (cytokines) [26]. In normal conditions, TDO is the main enzyme, but IDO is subject to induction during immune activation. The combined availability of IDO and TDO means that the overall capability of the kynurenine production is much increased. Therefore, serum TRP concentration can be reduced by 25%-50%, leaving proportionally less TRP available for conversion to serotonin [27, 28]. *In vivo* IDO activity can be measured similar to many enzymes by calculating the ratio of product against substrate (KYN/TRP). Thus a rise in ratio reflects greater activity; a reduction indicates lower activity. Our results indicate an induction of brain IDO following DS treatment.

NSAIDs produces their analgesic as well as anti-inflammatory effects through inhibition of cyclooxygenase (COX) enzyme 2. Cyclooxygenase

(COX) enzyme is responsible for the biosynthesis of the prostaglandins and certain related autacoids and is considered to be a major component of the mechanism of NSAIDs. Anti-inflammatory drugs achieve their therapeutic actions at least in part by regulation of cytokine formation. A “cytokine hypothesis” of depression is supported by the observation that depressed individuals have elevated plasma levels of certain cytokines compared with healthy controls. Depression is related to the levels of monoamines in the brain. Proinflammatory cytokines can affect monoamines regulation and uptake; thereby activate adrenal corticotrophin hormone and cortisol. The over expression of cortisol is the key link between the chronic stress and depression [28]. Prior studies also suggested that non-steroidal anti-inflammatory drugs (NSAIDs) may lower the incidence of Alzheimer's disease (AD) and delay onset or slow progression of symptoms in mouse models of AD [29]. Kynurenic acid (KYNA), an endogenous glutamate-receptor antagonist preferentially blocking NMDA-receptors, has analgesic properties and has also been implicated in the pathophysiology of schizophrenia. Diclofenac 50mg/kg was found to increase rat brain KYNA. It is proposed that increased brain KYNA formation induced by NSAIDs displaying an inhibitory action on COX-1 contribute to their analgesic efficacy as well as to their psychiatric side effects [30].

CONCLUSION

Our results show that DS inhibits hepatic TDO enzyme activity following chronic treatment, whilst augments brain IDO activity following both acute and chronic administration, that may result in the production of brain kynurenic acid and/or neuro-toxic metabolite, quinolinic acid therefore there is a need that effects of DS on kynurenine pathway should be further investigated to rule out the protective effect of DS in inflammation-induced depression and AD.

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