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Effects of 540C91 [(*E*)-3-[2-(4′-pyridyl)-vinyl]-1*H*-indole], an Inhibitor of Hepatic Tryptophan Dioxygenase, on Brain Quinolinic Acid in Mice

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ABSTRACT. Studies were undertaken to assess the role of the liver in the formation of the neurotoxin quinolinic acid in the brain. A selective and potent inhibitor of hepatic tryptophan 2,3-dioxygenase, 540C91 [(*E*)-3-[2-(4′-pyridyl)-vinyl]-1*H*-indole], largely prevented the elevation in mouse brain quinolinic acid resulting from parenteral injection of tryptophan (TRP). In contrast, 540C91 did not affect basal levels of the neurotoxin. Following induction of indoleamine dioxygenase with bacterial lipopolysaccharide, 540C91 was less effective in preventing the TRP-induced elevations in quinolinic acid. The data suggest that kynurenines, formed from tryptophan, by the liver and other extrahepatic organs can give rise to brain quinolinic acid. BIOCHEM PHARMACOL 51;2:159–163, 1996.

KEY WORDS. neurotoxins; quinolinic acid; NMDA receptor; kynurenines; tryptophan pyrrolase; tryptophan

QUIN§ is a neurotoxin formed *in vivo* from TRP. *In vivo*, exogenous quinolinate produces axon-sparing lesions following intra-striatal injection in rats [1]. *In vitro*, QUIN causes time-dependent pathology to neuronal cultures [2, 3].

QUIN is normally synthesized in the liver, via the kynurenine pathway (Fig. 1), as a precursor to the nicotinamide-containing cofactors [4, 5] and is also made by macrophages [6, 7]. The pathway for synthesizing QUIN from TRP begins with the oxidative decyclization of the pyrrole ring of TRP to yield kynurenine. In the liver, the reaction is catalyzed by TDO (EC 1.13.1.12), whereas in macrophages and elsewhere in the body the step is catalyzed by IDO (EC 1.13.11.17). The enzymes forming QUIN are found in the brain and in the liver; however, the liver contains far higher activities for all of these enzymes [8–11]. Since the blood–brain barrier is permeable to most of the QUIN precursors, but is relatively impermeable to QUIN [12, 13], it is possible that liver-derived kynurenines could be substrates for brain QUIN. We tested this possibility by preventing the hepatic formation of kynurenine with a novel, potent and selective inhibitor of hepatic TDO, 540C91 (one of a series of inhibitors of hepatic TDO synthesized at Wellcome). We found that inhibition of hepatic TDO prevented the TRP-induced increase in mouse brain QUIN.

MATERIALS AND METHODS

Male C57Bl/6 mice obtained from the Charles River Laboratories (U.K.) were allowed *ad lib.* access to food and water, in a temperature- and humidity-controlled facility on a 12-hr light/dark cycle. The animals were fasted overnight prior to use.

Reagents were obtained from Sigma (U.K.) unless otherwise indicated. TRP was prepared as a 0.1 M solution in phosphate-buffered saline by initially heating (95°) and subsequently maintaining the solution at 37° to maintain solubility. TRP was administered i.p. at a dose of 204 mg/kg in a volume of 10 mL/kg. The TDO inhibitor 540C91 was dissolved in saline and administered orally at 50 mg/kg in a volume of 10 mL/kg, 30 min prior to the TRP. Bacterial LPS, from *Salmonella typhimurium*, was administered i.p. in phosphate-buffered saline (4 mg/kg in an injection volume of 10 mL/kg) 24 hr before subsequent dosing with either TRP, 540C91, or a combination of both. Animals were killed by cervical dislocation 60 min after receiving TRP. Brain cortices were dissected free hand and stored at −70° until assay. Serum was collected at room temperature and stored at −20° until assay.

Cell-free assay of TDO was performed under conditions of linearity with time and enzyme concentration. The enzyme was isolated by homogenizing liver in 20 vol. of 0.05 M KPO_4,

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§ *Abbreviations:* QUIN, quinolinic acid; TRP, L-tryptophan; TDO, tryptophan 2,3-dioxygenase; IDO, indoleamine 2,3-dioxygenase; LPS, lipopolysaccharide; and 540C91, [(*E*)-3-[2-(4′-pyridyl)-vinyl]-1*H*-indole].

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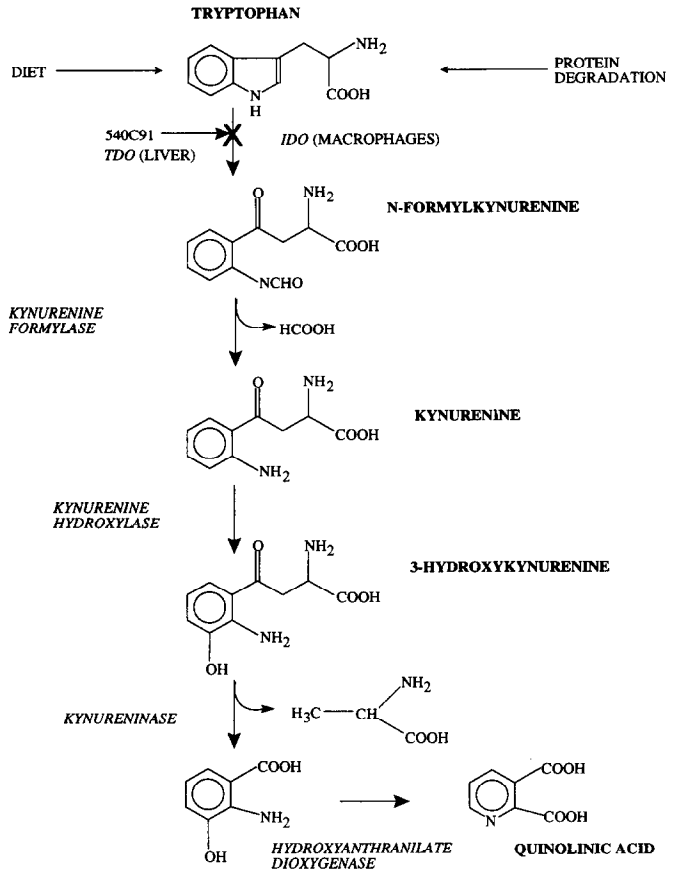

FIG. 1. The kynurenine pathway.

pH 7.0, containing 0.136 mg/mL methemoglobin at 4°. The homogenate was centrifuged at 100,000 *g* for 30 min, and the supernatant was used as the enzyme source. Assays were conducted, at 37°, in a total volume of 200 μL containing: KPO_4, pH 7.0, 50 mM; TRP (as indicated); methemoglobin, 0.136 mg/mL; and the inhibitor. The assays were terminated after 60 min by adding 0.1 vol. of 20% (v/v) $HClO_4$. The resulting suspensions were clarified by centrifugation at 12,000 *g* for 2 min, and 50-μL aliquots were assayed for kynurenine by HPLC [14]. The solid phase was a C_{18} column (4 × 80 mm Zorbax ODS, DuPont Institute, Wilmington, DE). The mobile phase consisted of 0.1 M acetic acid, adjusted to pH 4.5 with concentrated NH_4OH with 1% (v/v) methanol as the organic modifier, and was delivered at a flow rate of 2 mL/min. The kynurenine product was detected by UV absorption at 360 nm. The other kynurenine pathway enzymes were assayed as described previously: IDO was assayed radioenzymatically, using mouse epididymus [15]; kynurenine hydroxylase was assayed radioenzymatically, using a rat liver mitochondrial preparation [10]; kynureninase was assayed by liquid chromatography with fluorescence detection using rat liver cytosol [16]; and hydroxyanthranilate 3,4-dioxygenase was assayed radioenzymatically, with rat liver cytosol [17, 18].

Tissue contents of TRP and kynurenine were assayed by HPLC [14] following homogenization of the tissues in 0.1 M $HClO_4$ and centrifugation at 12,000 *g* × 20 min to remove insoluble protein. QUIN was determined using gas chromatography with mass spectrometry (GC/MS) in the negative chemical ionization mode, as described earlier [19] with slight modifications. Briefly, tissues were sonically disrupted in Tris · HCl (50 mM, pH 7.7) containing 60 nM $^{13}C_7$-QUIN (Cambridge Isotope Laboratories, Woburn, MA) as the internal standard and were denatured by heating (90° for 10 min). The supernatants (12,000 *g* × 10 min) were washed with an equal volume of chloroform, applied to anion exchange columns, eluted in 2 M formic acid, dried, converted to their bis-(hexafluoroisopropyl) esters and applied to the GC/MS.

Data were subjected to analysis of variance, and inter-group differences were assessed with the Tukey–Kramer test using the JMP® software for the Macintosh computer (SAS Institute, Cary, NC, U.S.A.). Probabilities <0.05 were considered significant.

RESULTS

The compound 540C91 competitively inhibited TDO with an apparent K_i of 240 nM (Fig. 2). In separate studies, 540C91 inhibited mouse epididymal IDO with an IC_{50} of 1.15 mM and, at 100 μM, did not inhibit kynurenine hydroxylase, kynureninase, or hydroxyanthranilate 3,4-dioxygenase (data not shown).

One hour after administration of TRP, serum TRP levels were not significantly different from vehicle-treated and control mice. Co-administration of 540C91 with TRP markedly increased TRP in both normal and LPS-treated mice (Fig. 3A). Administered by itself, 540C91 increased brain TRP by 28% but did not alter significantly brain levels of either kynurenine or QUIN (data not shown).

One hour after TRP, serum kynurenine levels were elevated markedly; no significant differences were observed between control mice and those that had received LPS. Co-administration of the TDO inhibitor with TRP prevented the rise in serum kynurenine in control mice and decreased the rise in LPS-treated mice (Fig. 3B).

Brain QUIN levels were elevated in control mice 1 hr after TRP. Larger increases in brain QUIN were seen in animals

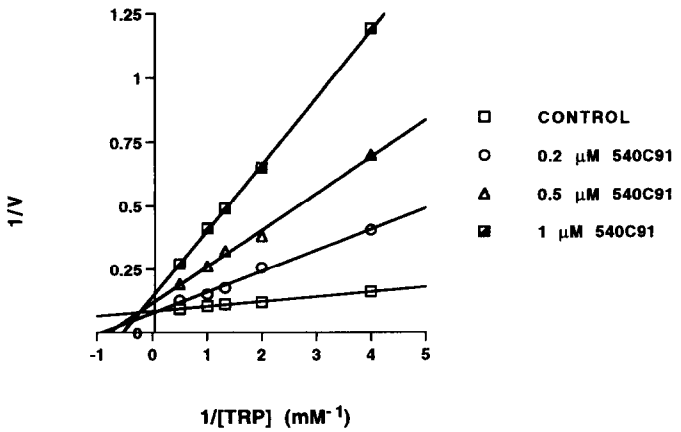

FIG. 2. Inhibition of TDO by 540C91. The units of velocity are expressed as nmol/(hr · mg tissue weight).

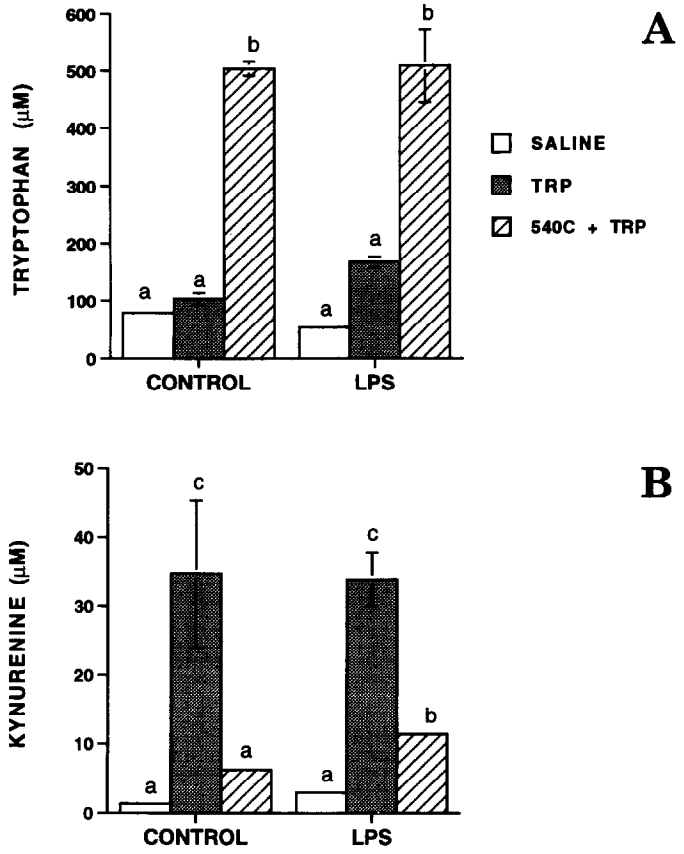

FIG. 3. Effects of TRP and 540C91 on serum TRP (A) and (B). The data are the means ± SEM of 5 animals per group. Groups with different letter superscripts differed significantly ($P < 0.05$). The TRP was administered i.p. at 204 mg/kg, while the 540C91 was given p.o. at 50 mg/kg. Animals were killed 1 h after TRP. LPS was administered at 4 mg/kg i.p. 24 hr before the TRP.

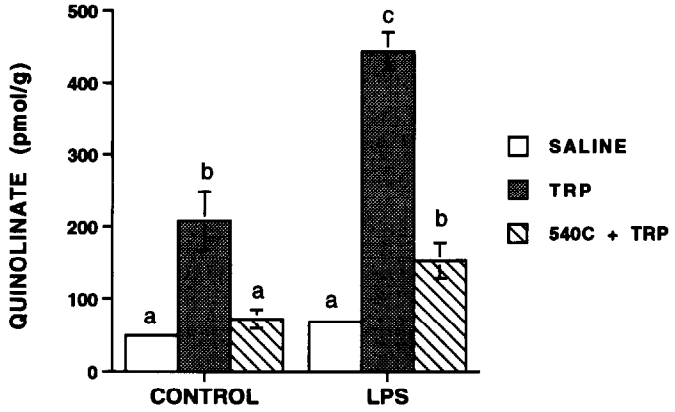

FIG. 4. Effects of TRP and 540C91 plus TRP on mouse brain QUIN. The Data are the means ± SEM of 5 animals per group. Groups with different letter superscripts differed significantly ($P < 0.05$). TRP was administered i.p. at 204 mg/kg, while 540C91 was given p.o. at 50 mg/kg. Animals were killed 1 hr after TRP. LPS was administered at 4 mg/kg i.p. 24 hr before the TRP.

that had been treated with LPS 24 hr earlier. Co-administration of 540C91 with TRP completely prevented the increase in brain QUIN in control mice and partially antagonized the increase in LPS-treated mice (Fig. 4). Nearly identical data were observed when rats were used (data not shown).

No changes were found in the levels of TRP, kynurenine, or QUIN in the serum 1 hr after a single, 50 mg/kg oral dose of the inhibitor.

DISCUSSION

The compound 540C91 is a potent and selective inhibitor of hepatic TDO. The data in Fig. 2 demonstrate that the inhibition of TDO was competitive with TRP, having a K_i of 240 nM. The effects of 540C91 seen *in vivo* in this study are unlikely to be the result of inhibiting other kynurenine pathway enzymes since concentrations of 540C91 as high as 100 μM did not inhibit significantly kynurenine hydroxylase, kynureninase, or hydroxyanthranilate dioxygenase. In addition, the low affinity of 540C91 for IDO (IC_{50} = 1.15 mM) makes it unlikely that 540C91 significantly inhibited IDO in these studies.

In vivo, the effects of 540C91 were evaluated in mice that had received TRP in a dose sufficient to elevate brain QUIN [19]. Moreover, the effects observed were consistent with inhibition of hepatic TDO. When 540C91 was co-administered with TRP, serum TRP levels were increased markedly. Moreover, the increase in serum kynurenine following TRP administration was abolished completely by 540C91 in control mice.

In the present studies, intraperitoneal injection of mice with TRP increased brain QUIN markedly. In earlier studies using rats, peripheral injection of TRP increased QUIN in both brain tissues [19] and microdialysates [13]. The increases in brain QUIN were largely in the brain parenchyma because saline exsanguination attenuated by only 40% the increase in brain QUIN [19]. Moreover, in a subsequent study, intravenous infusion of QUIN was largely ineffective at elevating the QUIN content of brain dialysates [13]. The present increases in brain QUIN, following TRP, would similarly be expected to contain a small vascular component. Since QUIN crosses the blood–brain barrier poorly [12], most of the TRP-induced increases may be attributed to QUIN synthesis in the brain. In an earlier study, QUIN was not detected in the dialysate when brain tissue was dialyzed with TRP. In contrast, the authors recovered QUIN following dialysis with 3-hydroxyanthranilic acid [20]. The failure of the latter study to detect QUIN in the dialysate, after perfusion of the brains with TRP, can be explained by the low activities of the other kynurenine pathway enzymes, apart from hydroxyanthranilate 3,4-dioxygenase, in rat brain.

Whereas liver requires TDO for TRP degradation, macrophage QUIN biosynthesis requires IDO (Fig. 1). Immune stimulation induces IDO [21] and increases the flux of kynurenine to QUIN [22–24]. In the present study, LPS was used to stimulate IDO. Following LPS, 540C91 was less effective in preventing the rise in serum kynurenine following TRP (Fig. 3B). The reduced effectiveness of 540C91 in LPS-treated mice can be explained by a rise in kynurenine, which would be caused

by the increase in IDO that is only weakly inhibited by 540C91.

TRP also increased brain QUIN in LPS-treated mice more than it did in control mice (Fig. 4). The greater increase in brain QUIN in LPS-treated mice cannot be explained by the levels of serum kynurenine. Alternatively, the augmented increase in brain QUIN in the LPS-treated mice can be explained by the induction of IDO in macrophages throughout the body. The kynurenine formed could then be converted to QUIN in the brain by either infiltrating or resident macrophages (microglia) [6, 25]. In LPS-treated mice, in contrast to control animals, co-administration of 540C91 with TRP only partially antagonized the increased QUIN (Fig. 4). The data can be explained by a two-site model for kynurenine formation, wherein both liver and macrophages contribute to kynurenine and QUIN formation through TDO- and IDO-dependent pathways, respectively.

The present data are consistent with the hypothesis that kynurenine, formed by the liver, can enter the brain where it is metabolized to QUIN. In support of this idea, kynurenine is transported from blood to brain by the large neutral amino acid transporter [12]. Moreover, peripheral administration of kynurenine increases brain QUIN [26]. Nevertheless, because of the low activities of kynurenine pathway enzymes in the brain, other than hydroxyanthranilate 3,4-dioxygenase, it is also possible that brain QUIN could result from the hepatic synthesis of other TRP metabolites such as 3-hydroxykynurenine or 3-hydroxyanthranilic acid. In support of this possibility, we have observed that the administration to mice of 3-hydroxykynurenine or 3-hydroxyanthranilic acid, in equimolar doses, elevated brain QUIN more than either TRP or any of its other kynurenine pathway metabolites [26]. Moreover, the data are not unique to the mouse since similar effects of kynurenine were demonstrated in primates [27]. As the inhibition of TDO, by administration of 540C91, will decrease the formation of all kynurenine pathway intermediates, the present data do not permit us to determine the relative contributions of these pathway intermediates for the synthesis of brain QUIN.

In conclusion, the liver pathway for TRP degradation can contribute to the increase in brain QUIN, following TRP administration, by increasing the concentration of precursors for brain QUIN synthesis. Activation of the immune system, with induction of IDO, can provide an alternative pathway for QUIN synthesis. The formation of QUIN *in vivo* is thus a consequence of multiple organ interactions where the contribution of each is state dependent.

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