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EXAMINATION OF MOUSE AND RAT TISSUES FOR EVIDENCE OF DUAL FORMS OF THE FATTY ACID CYCLOOXYGENASE

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Abstract—The possibility that the enzymatic generation of prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ results from the catalytic activity of two distinct forms of the fatty acid cyclooxygenase was studied in microsomes prepared from kidney, lung, and brain of the mouse and rat. Three criteria established previously to detect the dual cyclooxygenase forms in the rabbit brain were used in the present study: (1) different time course profiles of microsomal PGE_2 and $PGF_{2\alpha}$ biosynthesis from exogenous arachidonic acid; (2) elimination of the synthesis of one PG *in vitro* by non-steroidal anti-inflammatory drug concentrations that did not affect the synthesis of the other PG and; (3) selective autocatalytic inactivation of one cyclooxygenase by preincubation with arachidonic acid. Incubations with PGH_2 endoperoxide as substrate tested whether the altered PG biosynthesis resulted from an effect on the endoperoxide utilizing enzymes and not on the cyclooxygenase. Of the six tissues examined, only the mouse brain microsomes satisfied all the criteria. The microsomes prepared from the mouse kidney produced mixed results. We conclude that the mouse brain but not the rat brain gives evidence for two distinct forms of the fatty acid cyclooxygenase. Additional distinguishing features of the different cyclooxygenases are required to determine if the cyclooxygenase forms are found in mouse kidney.

The biosynthesis of the prostaglandins (PGs)‖ from arachidonic acid (AA) initially involves the conversion of the fatty acid to a cyclic endoperoxide intermediate by the membrane bound PG endoperoxide synthetase. The PGH_2 endoperoxide formed is, in turn, utilized by various isomerases or synthetases or may be reduced to produce the primary PGs or thromboxane [1].

The fatty acid cyclooxygenase catalyzing the initial enzymatic step of the synthetase complex has been reported to be purified to homogeneity from bovine and sheep seminal vesicles which suggests that these tissues may possess one predominant form of the enzyme [2, 3]. However, other investigators have suggested that multiple forms of the enzyme may exist. Smith and Lands [4] reported that two forms of the fatty acid cyclooxygenase are present in the acetone powder preparation from sheep vesicular gland. One enzyme form (termed Ea) requires hydroperoxides to support activity with its action suppressed by glutathione (GSH) peroxidase, while the second enzyme form (Eb) is less active, does not require hydroperoxides, and is unaffected by GSH peroxidase. Flower and Vane [5] have reported that different synthetase systems in different regions of the body show different sensitivities to non-steroidal anti-inflammatory agents. In their opinion the data were consistent with the hypothesis that more than one cyclooxygenase enzyme existed in their dog spleen and rabbit brain preparations. Maddox [6] reported that homogenates of sheep vesicular tissue normally produce PGE_2 and $PGF_{2\alpha}$ but, when preincubated with select anti-inflammatory drugs, PGE_2, but not $PGF_{2\alpha}$ synthesis was inhibited. However, these studies did not consider the possibility that the drugs may have acted upon the PGH_2 endoperoxide utilizing enzymes.

The proposal that two forms of the fatty acid cyclooxygenase could be present in select tissues was addressed by Lysz and Needleman [7]. In those studies, PGE_2 biosynthesis in rabbit brain microsomes was reported to be associated with one cyclooxygenase which was inhibited by low concentrations of non-steroidal anti-inflammatory drugs. $PGF_{2\alpha}$ production was associated with a biochemically distinct, less active cyclooxygenase which required higher levels of anti-inflammatory agents to inhibit its activity.

The present study of mouse and rat tissues was undertaken to determine whether the dual cyclooxygenases are a common feature of brain PG generation and also to assess whether a tissue rich in the dual cyclooxygenases could be identified to permit isolation and purification of the isoenzymes. The three criteria used by Lysz and Needleman [7] to detect the cyclooxygenases in the rabbit brain were used in the present study: (1) different time course profiles of microsomal PGE_2 and $PGF_{2\alpha}$ biosynthesis from exogenous arachidonate; (2) elimination of the synthesis of one product *in vitro* at inhibitor con-

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‖ Abbreviations: PG ( ), prostaglandin (specific prostaglandin); GSH, glutathione; AA, arachidonic acid; and BSA, bovine serum albumin.

centrations that did not affect the synthesis of the other PG; and (3) the autocatalytic inactivation of one cyclooxygenase form, but not the other, by preincubation of the microsomes with arachidonate.

MATERIALS AND METHODS

1-[^{14}C]Arachidonic acid (AA; 53 mCi/mmol), [^{3}H]PGE_2 (150 Ci/mmol) and [^{3}H]$PGF_{2\alpha}$ (160 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Unlabeled AA was obtained from Nu-Chek-Prep (Elysian, MN) and unlabeled PG standards from Upjohn Diagnostics (Kalamazoo, MI). Bovine serum albumin (fatty acid poor) was purchased from Calbiochem (LaJolla, CA). Epinephrine and reduced glutathione (GSH) were obtained from Sigma (St. Louis, MO), and silica gel G plates were purchased from Brinkmann (Grand Island, NY). Male Swiss Webster mice (25–30 g) and Sprague–Dawley rats (150–200 g) were obtained from the Charles River Breeding Farms (Charles River, NY). Animals were housed on the premises for at least 1 week prior to use with a 12-hr light–dark cycle at constant temperature (25°) and humidity (52%). 1-[^{14}C]PGH_2 endoperoxide was generated enzymatically by the method of Gorman *et al.* [8]. Indomethacin was a gift from Dr. Philip Davies (Merck, Sharp & Dohme, Rahway, NJ). Ferrous chloride 4-hydrate was obtained from J. T. Baker (Phillipsburg, NJ).

Preparation of albumin-treated microsomes. Rat or mouse kidney, lung, or brain were hand-homogenized in 4 vol. of cold 0.1 M potassium phosphate buffer containing 1% BSA (fatty acid poor) and 10 mM EDTA [7]. The homogenates were centrifuged at 8,000 *g* for 15 min, and the resulting supernatant fraction was centrifuged at 100,000 *g* for 60 min. The 100,000 *g* pellets (microsomes) were washed five times with 0.1 M potassium phosphate buffer and resuspended in the same buffer to a protein concentration between 3 and 8 mg/ml [9].

Microsomal incubations with labeled arachidonate and isolation of prostaglandins. Microsomes (0.3 to 0.8 mg protein) were incubated in 150 μl of 0.1 M potassium phosphate buffer, pH 7.4, containing epinephrine (1 mM), reduced GSH (1 mM) and 1-[^{14}C]AA (300,000 cpm; 24 μM). Incubations were performed at 37° for various times, and the reactions were terminated by the addition of 50 μl of 4 N formic acid. PGs were extracted three times into 2 vol. of ethyl acetate, the ethyl acetate fractions were pooled and evaporated to dryness under N_2, and the residue was resuspended in 50 μl of chloroform–methanol (2:1).

PGs were isolated by thin-layer chromatography in chloroform–methanol–acetic acid–water (90:8:1:0.8) [10]. PG standards were co-chromatographed and visualized by iodine vapor to identify the products. The thin-layer plates were subjected to autoradiography (Kodak X-ray AR film-6 day exposure), and the bands corresponding to the PG standards were cut and counted. PGE_2 and $PGF_{2\alpha}$ and small amounts of PGD_2 and hydroxy fatty acid were produced by the microsomal incubations. Recovery of the label found on the TLC plates was between 65 and 75% of the total radioactivity added to the incubation.

Microsomal incubations with 1[^{14}C]*PGH_2 endoperoxide as substrate.* Microsomes or 0.1 M potassium phosphate buffer were preincubated with either 10 μM indomethacin or 1 μg unlabeled AA for 10 min at room temperature. 1-[^{14}C]PGH_2 endoperoxide (100,000 cpm) was added, and the sample was incubated for an additional 2 min at 37°. Incubations were terminated by the addition of ferrous chloride (20 mM final concentration) which converts any unreacted PGH_2 endoperoxide into 12-hydroxyheptadecatrienoic acid (HHT) [11, 12]. The samples were acidified with formic acid, and the PGs were extracted into ethyl acetate and subjected to TLC and autoradiography as described above. Results are expressed as percent conversion of 1-[^{14}C]PGH_2 endoperoxide into labeled PG per mg protein. Boiled tissue samples served as incubation blanks with the bands corresponding to the PGs formed via spontaneous degradation of the unstable endoperoxide subtracted from the incubated samples to gain a true indication of PGH_2 endoperoxide metabolism.

Radioimmunoassay of PGE_2 and $PGF_{2\alpha}$. Microsomes were prepared and incubated under similar conditions to those noted above except that 1 μg of unlabeled AA was used to replace the 1-[^{14}C]AA. At different times during the incubations, either a 10-μl or a 50-μl aliquot was removed and added to 490 or 450 μl of ice-cold RIA buffer, respectively. A 50-μl aliquot was also withdrawn from select samples after the addition of the AA but before initiation of the incubations to serve as controls. The RIA for PGE_2 and $PGF_{2\alpha}$ was performed according to the method of Reingold *et al.* [13] using antisera generated in our laboratory [14].

Statistics. Statistical analyses were performed using Student's *t*-test for unpaired or paired samples.

RESULTS

Figure 1 profiles the time courses of PGE_2 and $PGF_{2\alpha}$ production in the microsomes prepared from the mouse and rat whole brain. Mouse brain PGE_2 biosynthesis, measured by RIA, was rapid and plateaued within 5 min following addition of the fatty acid substrate, while $PGF_{2\alpha}$ synthesis continued throughout the 60-min incubation period. The time course of rat brain $PGF_{2\alpha}$ biosynthesis paralleled the rate of PGE_2 formation with the production of both PGs essentially complete within a 10-min incubation. The continued rise in $PGF_{2\alpha}$ synthesis in the mouse brain microsomal preparations was not due to the conversion of PGE_2 via a 9-ketoreductase since separate incubations of the mouse brain microsomes with [^{3}H]PGE_2 did not produce any [^{3}H]-$PGF_{2\alpha}$ (data not shown). Feedback inhibition of the PGE_2 upon the $PGH_2 \rightarrow PGE_2$ isomerase was ruled out, since earlier studies demonstrated that addition of 1 μg PGE_2 to the incubation medium does not suppress [^{14}C]PGE_2 synthesis from labeled AA [7].

These initial studies suggested that the mouse brain might possess dual cyclooxygenase activities. The results obtained in rat brain indicated that not all brain tissues would satisfy the unique characteristics established to identify the presence of dual cyclooxy-

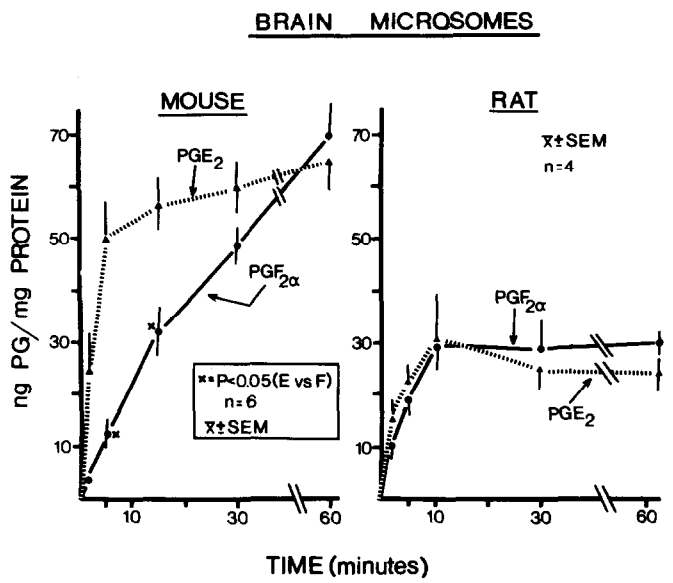

Fig. 1. Time course of PGE_2 and $PGF_{2\alpha}$ production in mouse and rat brain microsomes, measured by radioimmunoassay. Microsomes (0.3 to 0.8 mg protein) were incubated with reduced GSH (1 mM), epinephrine (1 mM) and unlabeled AA (1 μg; 24 μM) at 37°. The reactions were terminated by adding a 50-μl aliquot of the incubation mixture to 450 μl of ice-cold RIA buffer. Each value represents the means ± SEM with the number of determinations noted within the figure.

genases. These conclusions were strengthened by the results obtained following preincubation of the rat and mouse brain microsomes with various concentrations of indomethacin (Fig. 2). Mouse brain microsomal PGE_2 biosynthesis was decreased significantly ($P < 0.01$) at a concentration of indomethacin (0.3 μM) that had little effect on $PGF_{2\alpha}$ synthesis. The 50% inhibitory concentration (IC_{50}) for mouse brain PGE_2 synthesis was approximately 0.2 μM, whereas the IC_{50} for mouse brain $PGF_{2\alpha}$ formation was >10 μM. Rat brain PGE_2 and $PGF_{2\alpha}$ production were inhibited to the same extent in response to indomethacin pretreatment (IC_{50} = 3 μM).

The sudden cessation of mouse brain PGE_2 synthesis seen in Fig. 1 may have resulted from the

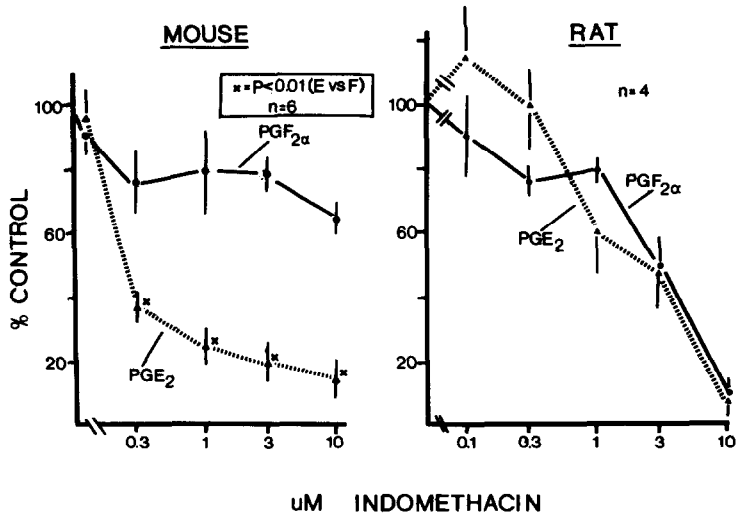

Fig. 2. Inhibition of mouse and rat brain prostaglandin biosynthesis by indomethacin. Microsomes prepared from either mouse (left panel) or rat (right panel) brain were preincubated for 5 min at room temperature with various concentrations of inhibitor. Following the preincubation period, the cofactors and unlabeled arachidonate were added, and the incubations were continued for 60 min at 37°. Values are the mean ± SEM of either four (rat) or six (mouse) determinations. One hundred percent control values for mouse and rat PGE_2 were 80.6 ± 11 and 14.7 ± 5 ng/mg protein respectively. Mouse and rat $PGF_{2\alpha}$ control values were 69 ± 13 and 25 ± 6 ng/mg protein.

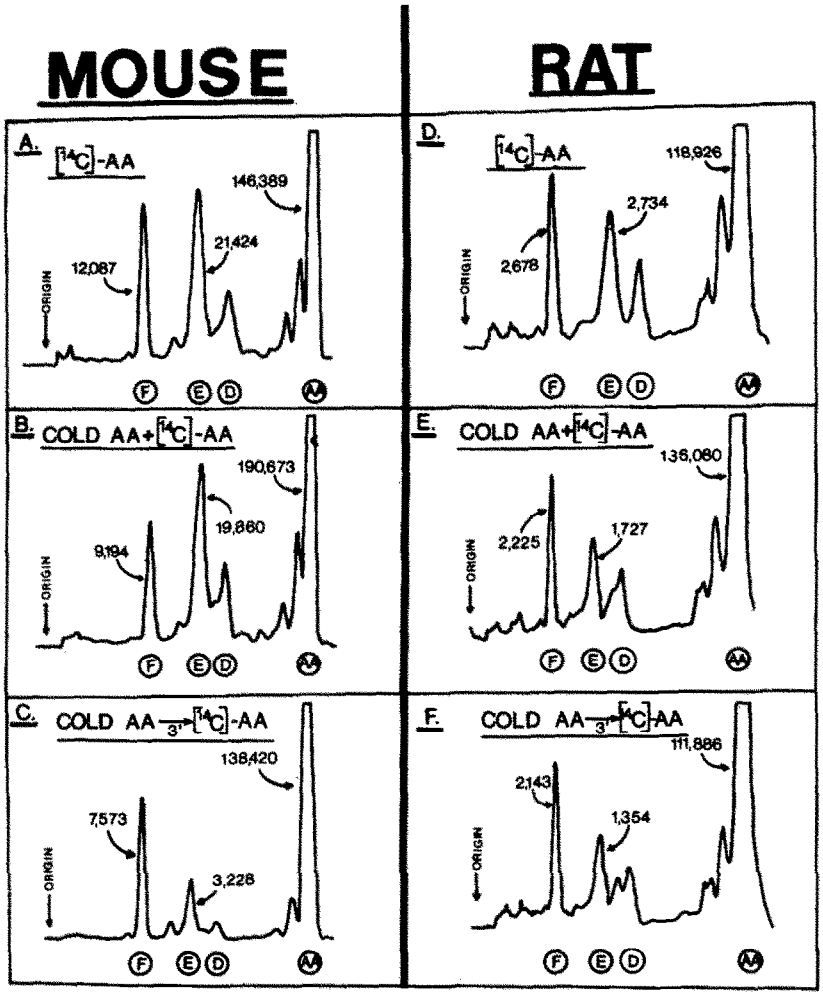

Fig. 3. Labeled PGE_2 and $PGF_{2\alpha}$ biosynthesis in rat and mouse brain microsomal preparations following preincubation with unlabeled arachidonate. Panels A and D: addition of 1-[^{14}C]AA (300,000 cpm; 1 μg) 3 min into the incubation of the mouse or rat brain microsomes. Panels B and E: the simultaneous addition of 1 μg of unlabeled AA and 1 μg 1-[^{14}C]AA 3 min into the incubation. Panels C and F: addition of 1 μg of unlabeled AA at the start (time zero) of the incubation period followed 3 min later by the addition of 1 μg 1-[^{14}C]AA. Microsomes were incubated for 60 min at 37° with reduced GSH (1 mM) and epinephrine (1 mM). Incubations were terminated by acidification, and the labeled PGs were isolated by TLC (see Materials and Methods). Following densitometry, the TLC plates were cut, and the bands corresponding to authentic PG standards were counted. The figure is representative of the data obtained from three separate experiments.

formation of hydroperoxides that are generated during the metabolism of AA [15]. To test this hypothesis, mouse and rat brain microsomes were incubated with 1 μg of unlabeled AA for 3 min at room temperature followed by a 60-min incubation with 1 μg 1-[^{14}C]AA. Mouse brain synthesis of labeled PGE_2 decreased dramatically, whereas labeled $PGF_{2\alpha}$ production was unaffected by the AA preincubation (Fig. 3, panels B and C). In contrast, the unlabeled AA preincubation with the rat brain microsomal preparations did not alter the synthesis of labeled PGE_2 or $PGF_{2\alpha}$ from labeled AA (Fig. 3, panels E and F). These results support the hypothesis that two fatty acid cyclooxygenases are present in mouse brain. One cyclooxygenase appears to be associated with PGE_2 synthesis and was sensitive to either the addition of AA or to an intermediate metabolite. A second fatty acid cyclooxygenase was associated with $PGF_{2\alpha}$ production and was resistant to inhibition by the AA preincubation. The rat brain microsomes showed no evidence of the dual cyclooxygenases.

Additional proof that the decreased mouse brain PGE_2 synthesis occurred at the level of the cyclooxygenase and not as the result of an inactivation of the $PGH_2 \rightarrow PGE_2$ isomerase was obtained with incubations of the mouse brain microsomes with 1-[^{14}C]PGH_2 endoperoxide (Table 1). The proportions of PGE_2:$PGF_{2\alpha}$:PGD_2 produced by the non-enzymatic breakdown of the PGH_2 endoperoxide in phosphate buffer was 2.8:1.0:1.4. The addition of the microsomal protein in the presence of GSH, a required cofactor for the $PGH_2 \rightarrow PGE_2$ isomerase

Table 1. Mouse brain microsomal $PGH_2 \rightarrow PGE_2$ isomerase activity using 1-[^{14}C]PGH_2 endoperoxide as substrate

	$PGF_{2\alpha}$	PGE_2	PGD_2	HHT
		(% of radiolabel recovered)		
Control*	4.8 ± 0.2	41.1 ± 0.2	14.4 ± 0.2	26.1 ± 0.2
Indomethacin†	6.1 ± 0.6	41.1 ± 1.5	12.0 ± 0.6	26.6 ± 0.9
Arachidonate‡	6.5 ± 0.5	40.0 ± 0.5	11.3 ± 0.5	27.1 ± 0.3
Buffer§	10.2 ± 0.4	28.5 ± 0.1	14.4 ± 0.1	32.1 ± 0.6

Microsomes (150 μl) were incubated for 2 min at 37° in the presence of reduced GSH (1 mM) and epinephrine (1 mM) with 100,000 cpm 1-[^{14}C]PGH_2 endoperoxide. Reactions were terminated by the addition of ferrous chloride (20 mM final concentration) which instantly converts any unreacted PGH_2 endoperoxide into 12-L-hydroxyheptadecatrienoic acid (HHT). Values are the percentage of the radiolabel recovered migrating with authentic PG standards and represent the mean ± SEM of three experiments. The balance of the radioactivity was found in the regions between the PGs on the TLC plate.

* Microsomes were incubated for 10 min at room temperature prior to the addition of arachidonate.

† Microsomes were preincubated for 10 min with 10 μM indomethacin before substrate addition.

‡ Microsomes were preincubated with 1 μg AA for 10 min at room temperature.

§ Potassium phosphate buffer (0.1 M, pH 7.4) was used instead of microsomes.

[16], shifted the proportions of PG production in favor of PGE_2 synthesis over the other PGs (4.1:0.6:1.0 for PGE_2, $PGF_{2\alpha}$ and PGD_2 respectively). Neither the addition of 10 μM indomethacin nor a 10-min preincubation of the microsomes with 1 μg of unlabeled AA affected the synthesis of PGE_2 or $PGF_{2\alpha}$ produced by the mouse brain microsomes during the incubation with PGH_2 endoperoxide.

The microsomal preparations from rat kidney and lung and from mouse lung were analyzed for evidence of the presence of the dual cyclooxygenases. None of the criteria were satisfied by the microsomal preparations from these tissues. However, the results obtained from the mouse kidney microsomal preparations satisfied two of the three criteria (Fig. 4). In the time course study, PGE_2 synthesis from exogenous AA ceased abruptly after 2 min, while $PGF_{2\alpha}$ production continued through the 60-min incubation period. In addition, a differential inhibition by indomethacin of mouse kidney microsomal synthesis of PGE_2 (IC_{50} = 0.6 μM) and $PGF_{2\alpha}$ (IC_{50} = 5 μM) was observed. Preincubating the mouse kidney microsomes with unlabeled AA for 3 min, however, failed to eliminate 1-[^{14}C]PGE_2 synthesis from 1-[^{14}C]AA (Table 2B). Radiolabeled mouse kidney PGE_2 and $PGF_{2\alpha}$ production was decreased to the

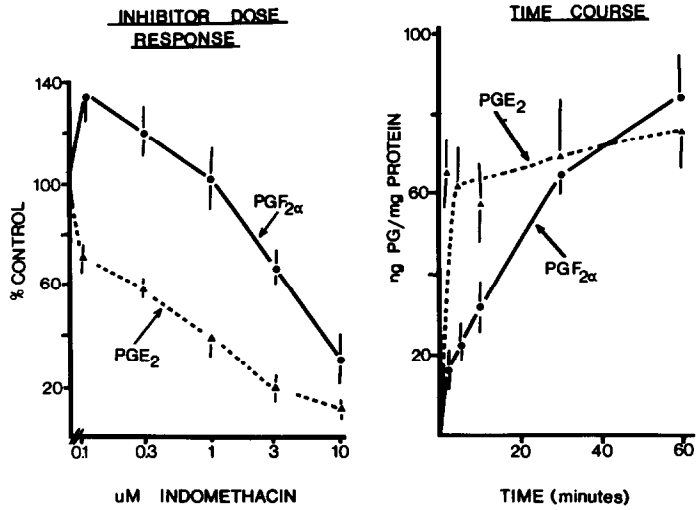

Fig. 4. Differential inhibition of prostaglandin E_2 and $PGF_{2\alpha}$ and the time course of eicosanoid production in mouse kidney microsomes. Left panel: microsomes were preincubated with various concentrations of indomethacin at room temperature for 5 min before addition of 1 μg AA. Samples were then incubated for an additional 60 min, and eicosanoid levels were determined by RIA. Each value represents the mean ± SEM of between five and six determinations. One hundred percent (control) values were 68 ± 15 and 125 ± 12 ng/mg protein for $PGF_{2\alpha}$ and PGE_2 respectively. Right panel: time course of PGE_2 and $PGF_{2\alpha}$ production from AA. Each value is the mean ± SEM of four determinations.

Table 2. Comparison of PGE_2 and $PGF_{2\alpha}$ production in mouse kidney and brain following preincubation with unlabeled arachidonic acid

Treatment	Mouse kidney		Mouse brain	
	PGE_2 (ng [^{14}C]PG/mg protein)	$PGF_{2\alpha}$	PGE_2 (ng [^{14}C]PG/mg protein)	$PGF_{2\alpha}$
(A)* [^{14}C]AA alone at 3 min	75 ± 15 (170)	114 ± 35 (208)	60 ± 5 (139)	46 ± 5 (135)
(B)† AA (0–3 min) then [^{14}C]AA at 3 min	27 ± 20 (39)	35 ± 17 (36)	6 ± 1 (86)	25 ± 7 (25)
(C)‡ AA +[^{14}C]AA together at 3 min	44 ± 13 (100)	55 ± 18 (100)	43 ± 6 (100)	34 ± 15 (100)

Microsomes were incubated using the conditions described in Fig. 3, panels A through C. Values are the mean ± SEM of three separate experiments and denote the ng labeled PG/mg protein after a 60-min incubation. The number in parentheses represents the percent change from control (treatment C).

* (A) Labeled AA (1 μg) was added 3 min into the 37° incubation.

† (B) Unlabeled AA (1 μg) was added at the beginning of the microsomal incubation (time = 0) followed 3 min later by the addition of 1-[^{14}C]AA (1 μg).

‡ (C) Unlabeled AA (1 μg) and 1-[^{14}C]AA (1 μg) were added simultaneously 3 min into the incubation.

same extent (approx. 60–65% of control) following preincubation of the kidney microsomes for 3 min with the unlabeled AA and incubation with labeled AA. In contrast, mouse brain microsomal PGE_2 synthesis, examined under identical incubation conditions, decreased 87% compared to a 25% decline in $PGF_{2\alpha}$ production.

DISCUSSION

The results obtained are consistent with the hypothesis that dual cyclooxygenases are present in the mouse brain. One cyclooxygenase is apparently associated with PGE_2 biosynthesis and inhibited with a short incubation with AA, while a second, biochemically distinct, enzyme is associated with $PGF_{2\alpha}$ production and resistant to inhibition by AA.

We considered several possible explanations for the results obtained from the mouse brain microsomes. The presence of a 9-ketoreductase, which can convert PGE_2 into $PGF_{2\alpha}$, could have explained the continued rise in $PGF_{2\alpha}$ synthesis after the sudden termination of PGE_2 production. However, the 9-ketoreductase is a cytosolic enzyme requiring NADPH as cofactor [17, 18]. No reductase activity was measured in the brain microsomal preparations. The decreased mouse brain PGE_2 synthesis measured after preincubation of the microsomes with AA could have resulted from inhibition of PG synthesis at the level of the $PGH_2 \rightarrow PGE_2$ isomerase. However, incubations with PGH_2 endoperoxide established that neither the addition of indomethacin nor the preincubation with AA inhibited the PGE_2 isomerase enzyme. These results, together with the select inhibition of PGE_2 synthesis by low concentrations of indomethacin, indicate that the alterations in PG production occurred at the level of the cyclooxygenase.

While the present results involving the mouse brain and the previous study of rabbit brain [7] suggest that dual cyclooxygenases are present in brain, final proof awaits the isolation and purification of each isozyme. Brain tissue contains limited amounts of the cyclooxygenase enzyme and extensive purification procedures, as described by others [3], are unlikely to succeed. The survey of extraneuronal tissues was performed as an attempt to identify a rich source of the cyclooxygenase for purification purposes.

The reasons for mouse brain PG synthesis to utilize the dual cyclooxygenases, while a single enzyme form suffices for PG synthesis in the rat brain, are unknown. Responses elicited by the PGs or their metabolism may indicate species differences. For example, PGE_2 inhibits the release of norepinephrine from the guinea pig vas deferens but not in rat [19]. Rat cerebral cortex adenylate cyclase is stimulated by PGE_2 but the eicosanoid has no effect on the rabbit cortical adenylate cyclase enzyme [20]. Differences in PG sensitivity between the noradrenaline neurons of the rat and rabbit brain cortex have been reported [21]. The lack of PG receptors in the rabbit brain cortex was offered as a possible explanation for the different effects observed between the two species. Differences in the capabilities of the rat and mouse brain to synthesize PGs may help explain the different effects of the non-steroidal anti-inflammatory agents on drug-induced seizure thresholds in the rat and mouse. Wallenstein and Mauss [22] have reported that the administration of the cyclooxygenase inhibitors ibuprofen, mefenamic acid, and meclofenamate increases the latency to the first epileptiform burst in rats and increases the time until the first convulsion. Steinhauer *et al.* [23] and McGinley *et al.* [14] have reported that inhibition of mouse brain cyclooxygenase activity by non-steroidal anti-inflammatory drugs lowers the threshold for pentylenetetrazol-induced convulsions. The decreased production of an anticonvulsant PGE_2 in the mouse brain occurring as a result of treatment with cyclooxygenase inhibitors may be a significant factor in determining the response of an animal to convulsant challenge.

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