Transformation of Arachidonate into 6-Oxoprostaglandin $F_{1\alpha}$, Thromboxane B_2 and Prostaglandin E_2 by Sheep Lung Microsomal Fraction

By HSIN-HSIUNG TAI,*† BARBARA YUAN* and AGNES T. WU†

*Department of Medicine, The Genesee Hospital and the University of Rochester School of Medicine
and Dentistry, Rochester, NY 14607, U.S.A., and

†LKB Instruments Inc., Rockville, MD 20852, U.S.A.

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In the presence of haemoglobin and isoproterenol, the microsomal fraction of sheep lung catalysed the conversion of arachidonate predominantly into thromboxane B_2 and to a lesser extent into 6-oxoprostaglandin $F_{1\alpha}$. Very little prostaglandin E_2 and prostaglandin $F_{2\alpha}$ were formed. If reduced glutathione was added in combination with haemoglobin and isoproterenol, the synthesis of prostaglandin E_2 was favoured over that of thromboxane B_2 and 6-oxoprostaglandin $F_{1\alpha}$. The identities of these products were confirmed by t.l.c. and by combined g.l.c.–mass spectrometry. These results indicate that microsomal fraction of sheep lung possesses active prostaglandin synthase, prostacyclin synthase and thromboxane synthase activities.

Mammalian lung has been known for more than a decade to transform arachidonate into prostaglandins (Anggard, 1965). Hamberg & Samuelsson (1974a) described the novel transformation of arachidonate into a non-prostanoate product, thromboxane B_2 [8-(1-hydroxy-3-oxopropyl)-9,12L-dihydroxyheptadeca-5,10-dienoic acid], in guinea-pig lung. This compound was subsequently found to be the hydrolysed product of a highly unstable intermediate designated thromboxane A_2 (Hamberg *et al.*, 1975). This unstable intermediate is now thought to be the major component of the 'rabbit aorta-contracting substance' previously described by Piper & Vane (1969) and is capable of inducing irreversible platelet aggregation (Hamberg *et al.*, 1975; Svensson *et al.*, 1975). Dawson *et al.* (1976) isolated 6-oxoprostaglandin $F_{1\alpha}$ (9 α ,11 α ,15 α -tri-hydroxy-6-oxoprost-13-*trans*-enoic acid) in addition to thromboxane B_2 and prostaglandins and their catabolites from sensitized guinea-pig lungs after perfusion with antigen. The new compound, 6-oxoprostaglandin $F_{1\alpha}$, was proved to be the stable product of another unstable intermediate termed prostacyclin, which inhibited platelet aggregation (Moncada *et al.*, 1976; Johnson *et al.*, 1976). Apparently mammalian lung has the versatility of producing three classes of diversified biologically active substances from arachidonate. To understand the mechanism and the regulation of the biosynthesis of

these substances, clearly the enzyme systems that bring about these transformations must be isolated and characterized. The present paper demonstrates that arachidonate is transformed into the stable products 6-oxoprostaglandin $F_{1\alpha}$, thromboxane B_2 and prostaglandin E_2 (11 α ,15 α -dihydroxy-9-oxoprost-5-*cis*,13-*trans*-dienoic acid) by a sheep lung microsomal preparation.

Materials

Arachidonic acid, sheep haemoglobin, DL-isoproterenol {3,4-dihydroxy- α -[(isopropylamino)-methyl]benzyl alcohol} and GSH were from Sigma Chemical Company, St. Louis, MO, U.S.A. [1- 14 C]-Arachidonic acid was from New England Nuclear, Boston, MA, U.S.A. NO-Bis(trimethylsilyl)trifluoroacetamide and methoxyamine hydrochloride were from Pierce Chemical Company, Rockford, IL, U.S.A. Prostaglandin D_2 (9 α ,15 α -dihydroxy-11-oxoprost-5-*cis*,13-*trans*-dienoic acid) and thromboxane B_2 were kindly given by Dr. John Pike and Dr. Udo Axen of the Upjohn Company, Kalamazoo, MI, U.S.A. 6-Oxoprostaglandin $F_{1\alpha}$ was biosynthesized by the published procedures (Moncada *et al.*, 1976; Johnson *et al.*, 1976) by using pig aorta microsomal fraction as the enzyme source. The purified product showed one spot on t.l.c. in two solvent systems as described below and exhibited a mass spectrum identical with that reported in the literature for 6-oxoprostaglandin $F_{1\alpha}$ (Dawson *et al.*, 1976; Pace-Asciak, 1976; Chang *et al.*, 1976). Precoated silica-gel G plates were from Brinkmann Instruments, Westbury, NY, U.S.A. Sheep lungs were collected from a local slaughterhouse and stored at -40°C .

Abbreviation used: GSH, reduced glutathione.

† To whom reprint requests should be addressed at Department of Biochemistry, Texas College of Osteopathic Medicine, North Texas State University, Denton, TX 76203, U.S.A.

Methods

Preparation of microsomal fraction from sheep lung

Frozen sheep lung (100 g) was chopped into pieces and homogenized in a Waring Blendor operated at full speed for 2 min in 2 vol. of 0.05 M-potassium phosphate buffer, pH 7.5, containing 1 mM-EDTA. The suspension was centrifuged at 8000 g for 10 min in a Sorvall RC-5 refrigerated centrifuge. The supernatant was further centrifuged at 105 000 g for 60 min in a Beckman model L3-50 ultracentrifuge. The resulting pellet, designated as microsomal fraction, was homogenized in a glass homogenizer in 25 ml of the same buffer and stored in batches at -76°C .

Enzyme assays

The incubation mixture contained [$1\text{-}^{14}\text{C}$]arachidonic acid (33 nmol, 60 000 c.p.m.), sheep haemoglobin (1 nmol), isoproterenol (1 μmol) and microsomal fraction in a final volume of 1 ml of 0.1 M-potassium phosphate buffer, pH 7.5, containing 1 mM-EDTA. In cases where prostaglandin E_2 formation was to be observed, GSH (1 μmol) was also included in the incubation mixture. The incubation was carried out at 37°C for 5 min before termination of the reaction by acidification with 12 M-formic acid to pH 3.0. The reaction mixture was extracted with 2×3 ml of ethyl acetate. The organic layer was dried under N_2 and the residue, dissolved in acetone, was spotted on a silica-gel G plate (2 cm $\times$ 20 cm). The plate was developed in the following systems: (I) benzene/dioxan/acetic acid (20:10:1, by vol.) or (II) ethyl acetate/acetic acid/iso-octane (2,2,4-trimethylpentane)/water (11:2:5:10, by vol.). The bands corresponding to each prostaglandin and thromboxane B_2 were located by the aid of co-chromatography with authentic compounds and detection in iodine vapour.

Large-scale biosynthesis and isolation of reaction products from arachidonate

For isolation of various products for structural studies, 5 mg of arachidonic acid with added [$1\text{-}^{14}\text{C}$]arachidonic acid (0.3 μCi) as a tracer was incubated with sheep lung microsomal fraction (256 mg of protein) in the presence of isoproterenol (1 mM) and haemoglobin (1 μM) in a final volume of 100 ml of 0.1 M-potassium phosphate buffer, pH 7.5, containing 1 mM-EDTA, for 10 min at 37°C . The reaction mixture was acidified as above to pH 3.0 and extracted with 2×100 ml of ethyl acetate. The combined ethyl acetate extract was dried over Na_2SO_4 and evaporated to dryness. The residue was chromatographed on a silica-gel G plate (20 cm $\times$ 20 cm) in solvent system (I). The areas corresponding to 6-oxoprostaglandin

$\text{F}_{1\alpha}$, thromboxane B_2 , prostaglandin E_2 and prostaglandin D_2 were scraped off the plate and extracted with ethanol. The ethanol extracts were dried and derivatives prepared for g.l.c.-mass spectrometric analysis.

Preparation of derivatives

(a) *Methylation*. Diazomethane was generated from 200 mg of *N*-nitrosomethylurea in 2 ml of 50% (w/v) KOH and 4 ml of diethyl ether at 0°C . The purified extract was methylated with 0.5 ml of the ether layer and placed in the dark at room temperature for 1 h. The solvent was then evaporated to dryness by a stream of N_2 .

(b) *Methoxime formation*. A few micrograms of methyl esters of various keto compounds were converted into methoxime derivatives by treatment with 50 μl of 2% (w/v) methoxyamine hydrochloride in pyridine at 60°C for 1 h. The pyridine was evaporated by a stream of N_2 .

(c) *Trimethylsilylation*. Trimethylsilyl ether derivatives were prepared by dissolving a few micrograms of methyl esters of methoxime derivatives in 5 μl of *NO*-bis(trimethylsilyl)trifluoroacetamide at room temperature for 10 min. Samples were injected directly into the gas chromatograph.

G.l.c.-mass spectrometry

An LKB 2091 combined gas chromatograph-mass spectrometer equipped with an SE-30 glass capillary column (LKB 2101-303; 25 m in length, 0.33 mm internal diameter) was used. The column temperature was maintained at 200°C for 5 min and then programmed to increase at $10^{\circ}\text{C}/\text{min}$ to 300°C . Both the separator and the ion-source temperatures were kept at 180°C . The ionization voltage was maintained at 70 eV. The carrier gas was helium, with a flow rate of 2 ml/min.

Results

When incubated in the presence of isoproterenol and haemoglobin the microsomal fraction of sheep lung catalyses the transformation of [$1\text{-}^{14}\text{C}$]arachidonic acid into one major polar product C and one minor polar product A, as shown in Fig. 1(a). The major product C has the same mobility on a silica-gel G plate as authentic thromboxane B_2 in the two different solvent systems. The minor product A was originally thought to be prostaglandin $\text{F}_{2\alpha}$, because of its same mobility in chromatography system (I), which gave good separation of thromboxane B_2 from other prostaglandins. When the t.l.c. plate was developed in solvent system (II) product A appeared to be slightly more polar than prostaglandin $\text{F}_{2\alpha}$. Double development of the t.l.c. plate in the

Fig. 1. Thin-layer radiochromatogram of products isolated after incubation of $[1-^{14}\text{C}]$ arachidonic acid with sheep lung microsomal fraction

(a) $[1-^{14}\text{C}]$ Arachidonic acid was incubated with sheep lung microsomal fraction (2.6 mg of protein) in the presence of haemoglobin and isoproterenol as described in the Methods section. The ethyl acetate extract was chromatographed in the solvent system (I). Strips of the chromatogram were scraped off the t.l.c. plate and the radioactivity was determined by liquid-scintillation counting. (b) Incubation was carried out in the same manner as in (a) except that the extract was chromatographed in solvent system (II) for 17 cm. The plate was dried in air and redeveloped in the same solvent system for 12 cm. (c) Incubation and chromatography were carried out in the same manner as in (a) except that GSH was added in combination with haemoglobin and isoproterenol during incubation. Abbreviations: 6-oxo- $\text{F}_{1\alpha}$, 6-oxo-prostaglandin $\text{F}_{1\alpha}$; $\text{F}_{2\alpha}$, E_2 , D_2 , prostaglandins $\text{F}_{2\alpha}$, E_2 and D_2 respectively; B_2 , thromboxane B_2 .

same solvent system resulted in complete separation of the product A from prostaglandin $\text{F}_{2\alpha}$, and the product A appeared to co-chromatograph with 6-oxoprostaglandin $\text{F}_{1\alpha}$, as shown in Fig. 1(b). Since little prostaglandin $\text{F}_{2\alpha}$ was formed, the solvent system (II) was routinely used to monitor the synthesis of 6-oxoprostaglandin $\text{F}_{1\alpha}$, thromboxane B_2 and prostaglandin E_2 from arachidonic acid. When the incubation mixture was further supplemented with GSH, a new product, B, which appeared to be the major product, was formed, as shown in Fig. 1(c). The formation of this new product B was somewhat at the expense of product C, but not that of product A.

The product B had the same mobility as prostaglandin E_2 in the two solvent systems described above. A small but consistent product D, which appeared to be less polar than product C, also became apparent. Product D co-chromatographed with authentic prostaglandin D_2 in the two different solvent systems.

To identify each of the products, large-scale biosynthesis of these products was carried out and each of the products was purified and derivatives were formed for g.l.c.-mass spectrometric analysis. The mass spectrum of the methoximetrimethylsilyl ether derivative of the methyl ester of product A showed base peak at m/e 598. Other ions at m/e 629 (M^+), 614, 558, 539, 508, 468, 449, 418, 378, 353, 328, 322, 173 and 115 corresponded to those reported in the literature for 6-oxoprostaglandin $\text{F}_{1\alpha}$ (Dawson *et al.*, 1976; Pace-Asciak, 1976; Chang *et al.*, 1976). The g.l.c. data on the methoximetrimethylsilyl ether derivative of the methyl ester of product B showed the characteristic two peaks, presumably *syn*- and *anti*-isomers. The mass spectra of two isomers gave base peaks at m/e 468 and 225 respectively. Other ions at m/e 539 (M^+), 524, 508, 449, 418, 378, 365, 308, 199 and 173 corresponded exactly to those reported by Green (1969) for prostaglandin E_2 . The mass spectrum of product C gave a base peak at m/e 256 and other ions at 600 (M^+), 585, 510, 439, 420, 366, 323, 295, 225, 217 and 173, which were identical with those reported in the literature for thromboxane B_2 (Hamberg & Samuelsson, 1974a,b). The mass spectrum of product D was not successfully obtained, because of the limited quantity isolated and the unstable nature of the compound.

Discussion

The present paper demonstrates that the microsomal fraction of sheep lung possesses enzyme activities that catalyse the synthesis of 6-oxoprostaglandin $\text{F}_{1\alpha}$, thromboxane B_2 and prostaglandin E_2 from arachidonate to an appreciable extent. The microsomal fraction is known to exhibit low intrinsic enzyme activities (Takeguchi *et al.*, 1971; Tai *et al.*, 1976); however, the activity can be stimulated by haemoglobin (Yoshimoto *et al.*, 1970) and by phenolic and indole compounds (Nugteren *et al.*, 1966; Samuelsson, 1967; Sih *et al.*, 1970). These compounds have been shown to stimulate the prostaglandin endoperoxide synthase (fatty acid cyclo-oxygenase) (EC 1.14.99.1), which catalyses the formation of prostaglandin endoperoxides, the immediate precursors of prostacyclins, thromboxanes and prostaglandins (Miyamoto *et al.*, 1976; Hemler *et al.*, 1976; Tai, 1976). Therefore in the presence of haemoglobin and isoproterenol the microsomal fraction of sheep lung catalyses effectively the formation of endoperoxide for further transformation. The major product of such transformation

was identified as thromboxane B₂, on the basis of chromatographic properties on the t.l.c. plate and the identical mass spectrum with that of thromboxane B₂. The minor product was similarly identified as 6-oxoprostaglandin F_{1α}. The formation of thromboxane B₂ as the major product in lung tissue has been demonstrated by Hamberg & Samuelsson (1974a), using crude homogenates of guinea-pig lung. However, they failed to detect the formation of 6-oxoprostaglandin F_{1α}. Parkes & Eling (1974) also described the transformation of arachidonate by the microsomal fraction of guinea-pig lung. They found that the major product was a new prostaglandin derivative whose structure could not be identified. It is very likely that the new product described was actually thromboxane B₂. The other minor product found was claimed to be prostaglandin F_{2α}. However, the identification of this product was merely based on co-chromatography with prostaglandin F_{2α} in the solvent system (I), which they also used. It is necessary that the structure should be confirmed by other analytical means such as g.l.c.-mass spectrometric analysis.

Addition of GSH in combination with haemoglobin and isoproterenol apparently stimulated the formation of prostaglandin E₂ in favour of thromboxane B₂ and of 6-oxoprostaglandin F_{1α}. This was consistent with the fact that GSH augmented the synthesis of prostaglandin E₂ at the expense of other prostaglandins by stimulation of prostaglandin E synthase (isomerase) (Lands *et al.*, 1971; Foss *et al.*, 1971; Ogino *et al.*, 1976). The relative proportions of three different products apparently were determined by the respective enzyme concentrations, reaction rates and cofactor concentrations of prostaglandin E synthase, thromboxane synthase and prostacyclin synthase (isomerases). The presence of these three enzyme activities in sheep lung microsomal fraction provides a unique system to study the regulation of the synthesis of three different biologically active substances by various modulators. Furthermore, the availability of a large quantity of the microsomal preparation from sheep lung makes it more practical than platelet or guinea-pig system for further purification and elucidation of the organization and mechanism of the enzyme systems. In this regard, we have successfully solubilized the microsomal fraction from sheep lung and resolved the enzyme complex into prostaglandin endoperoxide synthase (fatty acid cyclo-oxygenase) and thromboxane synthase (isomerase) components (Tai & Yuan, 1977).

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References

- Anggard, E. (1965) *Biochem. Pharmacol.* **14**, 1507-1516
- Chang, W. C., Murota, S., Matsuo, M. & Tsurufuji, S. (1976) *Biochem. Biophys. Res. Commun.* **72**, 1259-1264
- Dawson, W., Bott, J. R., Cockerill, A. F., Mallen, D. N. B. & Osborne, D. J. (1976) *Nature (London)* **262**, 699-702
- Foss, P., Takeguchi, C., Tai, H. H. & Sih, C. J. (1971) *Ann. N. Y. Acad. Sci.* **180**, 126-137
- Green, K. (1969) *Chem. Phys. Lipids* **3**, 254-272
- Hamberg, M. & Samuelsson, B. (1974a) *Biochem. Biophys. Res. Commun.* **61**, 942-949
- Hamberg, M. & Samuelsson, B. (1974b) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3400-3404
- Hamberg, M., Svensson, J. & Samuelsson, B. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2994-2998
- Hemler, M., Lands, W. E. M. & Smith, W. L. (1976) *J. Biol. Chem.* **251**, 5575-5579
- Johnson, R. A., Morton, D. R., Kinner, J. H., Gorman, R. R., McGuire, J. C., Sun, F. F., Whittaker, N., Bunting, S., Salmon, J., Moncada, S. & Vane, J. R. (1976) *Prostaglandins* **12**, 915-928
- Lands, W. E. M., Lee, R. & Smith, W. L. (1971) *Ann. N. Y. Acad. Sci.* **180**, 107-122
- Miyamoto, T., Ogino, N., Yamamoto, S. & Hayaishi, O. (1976) *J. Biol. Chem.* **251**, 2629-2636
- Moncada, S., Gryglewski, R., Bunting, S. & Vane, J. R. (1976) *Nature (London)* **263**, 663-665
- Nugteren, D. H., Beerthuis, R. K. & Van Dorp, D. A. (1966) *Rev. Trav. Chim. Pays-Bas Belg.* **85**, 405-419
- Ogino, N., Miyamoto, T., Yamamoto, S. & Hayaishi, O. (1976) *J. Biol. Chem.* **252**, 890-895
- Pace-Asciak, C. (1976) *J. Am. Chem. Soc.* **98**, 2348-2349
- Parkes, D. G. & Eling, T. E. (1974) *Biochemistry* **13**, 2598-2604
- Piper, P. J. & Vane, J. R. (1969) *Nature (London)* **223**, 29-35
- Samuelsson, B. (1967) *Prog. Biochem. Pharmacol.* **3**, 59-70
- Sih, C. J., Takeguchi, C. & Foss, P. (1970) *J. Am. Chem. Soc.* **92**, 6670
- Svensson, J., Hamberg, M. & Samuelsson, B. (1975) *Acta Physiol. Scand.* **94**, 222-228
- Tai, H.-H. (1976) *Biochem. J.* **160**, 577-581
- Tai, H.-H. & Yuan, B. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 309
- Tai, H.-H., Tai, C. L. & Hollander, C. S. (1976) *Biochem. J.* **154**, 257-264
- Takeguchi, C., Kohno, E. & Sih, C. J. (1971) *Biochemistry* **10**, 2372-2376
- Yoshimoto, A., Ito, H. & Tomita, K. (1970) *J. Biochem. (Tokyo)* **68**, 487-499