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INFLUENCE OF ENDOTOXIN ON ARACHIDONATE METABOLISM IN ISOLATED RABBIT PERITONEUM*

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Abstract—The serous membranes of the rabbit peritoneal cavity are tissues in which cyclo-oxygenase and lipoxygenase pathways of arachidonate metabolism can be studied simultaneously. After elaboration of the optimum conditions, the metabolism of two concentrations of arachidonic acid (AA) was studied in the presence and absence of endotoxin lipopolysaccharide (LPS) from *E. coli* O127:B8 (0.1 and 1.0 mg/ml). LPS suppressed the formation of radiolabelled cyclo-oxygenase products (predominantly prostacyclin) at the lower concentration of exogenous AA (0.8 μM), but not at the higher substrate concentration (34.5 μM). The biosynthesis of lipoxygenase metabolites, i.e. monohydroxy-eicosatetra-enoic acids (HETEs), was not influenced by LPS. These findings can be explained by an enhanced release of endogenous AA in the prostacyclin forming mesothelial cells in the presence of LPS. Measurements of the endogenous biosynthesis of prostacyclin supported this assumption.

Prostacyclin (PGI_2) has vasodilator properties and inhibits blood platelet aggregation in mammals [1], but not in birds [2]. It is formed in blood vessels from free arachidonic acid (AA) by the actions of cyclo-oxygenase and prostacyclin synthase, and hydrolyses spontaneously to 6-oxo-$PGF_{1\alpha}$ in buffers with an acidic or neutral pH [1]. In rabbits, arterial blood levels of 6-oxo-$PGF_{1\alpha}$ rise from undetectable in the control period to 1–2 ng/ml after administration of endotoxin lipopolysaccharide (LPS) [3]. The cyclo-oxygenase inhibitor indomethacin suppresses both the LPS-induced fall in aortic blood pressure and the elevation of arterial 6-oxo-$PGF_{1\alpha}$ in rabbits [4] and other species [5], suggesting that PGI_2 is involved in the pathophysiology of endotoxic shock [4, 5]. Therefore, we investigated whether LPS affected PGI_2 biosynthesis in an *in vitro* system.

For this purpose isolated rabbit peritoneum was selected, since it is a richer source of PGI_2 biosynthesis than the large blood vessels of the rabbit [6]. Moreover, it has been reported that LPS increases the formation of immunoreactive PGE in isolated rat mesentery, and this coincides with elevated intracellular cAMP levels and an increased permeability of the mesentery for macromolecules [7]. Mesothelial cells are probably the main source of PGI_2 in rabbit peritoneal membranes [8]. Monolayers of these squamous, polygonal epithelial cells cover the surface of all serous membranes, and in culture rabbit mesothelial cells also form PGI_2 in quantities similar to aortic endothelial cells. Mesothelial cells differ from the latter by their larger size and the lack of angiotensin converting enzyme activity (H. Neels and V. J. S. Van de Velde, personal communication).

In addition, several lipoxygenase products [6], predominantly 15-L-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) and even more of the corresponding linoleic acid metabolite, 13-L-hydroxy-9,11-octadecadienoic acid (13-HODE), together with smaller amounts of 12-HETE, 11-HETE and its linoleic acid-analogue 9-HODE [9] can be formed by rabbit peritoneal tissue, but not by mesothelial cells [8].

MATERIALS AND METHODS

Materials. Radioactive [1-^{14}C]arachidonic acid (56.6 mCi/mmole) and [5,8,9,11,12,14,15-^{3}H]-6-oxo-prostaglandin $F_{1\alpha}$ (100 Ci/mmole) were purchased from New England Nuclear (Dreieich, F.R.G.); arachidonic acid (AA), soybean lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.12.11.12) and tris(hydroxymethyl)aminomethane (Tris) were from Sigma (St. Louis, MO); precoated thin-layer chromatography plates (0.25 silica 60 F254) were from Merck (Darmstadt, F.R.G.); endotoxin (lipopolysaccharide W *E. coli*; serotype O127: B8, lot No. 662053) was from DIFCO Labs. (Detroit, MI). The following products were generous gifts: eicosatetraynoic acid (ETYA) from Dr. C. Harper, Roche Products Ltd. (Welwyn Garden City, U.K.); indomethacin (IM) from Merck, Sharp & Dohme (Brussels, Belgium); prostaglandins ($PGF_{2\alpha}$, PGE_2, PGD_2, PGA_2 and 6-oxo-$PGF_{1\alpha}$) and thromboxane B_2 (TXB_2) from Dr. J. Pike, Upjohn Company (Kalamazoo, MI). All other reagents were of analytical grade.

Tissue preparation. Rabbits (2–3 kg) were killed by a blow on the head and exsanguination. Pieces of peritoneal tissue (omentum maius, mesogastrium, mesenterium of ileum, coecum and colon, and mesohepaticum ventrale), devoid of macroscopically

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visible blood vessels and fatty tissue, were removed, cut into pieces of similar size and stored in Krebs' solution. For incubation, the tissue (3–6 pieces, wet weight about 30 mg) was placed in 0.8 ml ice-cold 50 mM Tris–HCl buffer (pH 7.4 at 37°) unless indicated otherwise.

Radiochemical experiments. Incubations were carried out in a final volume of 1 ml Tris–HCl buffer, containing tissue, LPS (0, 0.1 or 1.0 mg, added in 0.1 ml 0.9% NaCl) and arachidonic acid (0.8 nmole [1-^{14}C]AA, or 1.5 nmole [1-^{14}C]AA with 0, 1.6, 4.8, 13.1, 26.2, 52.4 or 105.2 nmole unlabelled AA). In each experiment a blank was included (30 mg boiled tissue), and all treatments were randomized. Immediately after AA addition, the reactions were started by placing the incubations in a shaking water bath (37°, 180 strokes per min) for different periods.

Reactions were stopped by cooling (0°) and acidification to pH 3 (2 M citric acid). After addition of carriers/standards (6-oxo-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGD_2, PGA_2, 15-HPETE and AA, 10 μg of each) and tissue removal, an aliquot (50 μl) was taken for the measurement of total radioactivity in the mixture, and the reaction products were extracted twice with 2 volumes of ethyl acetate. The combined organic phases were taken to dryness under a stream of nitrogen. The residue was redissolved in 50 μl chloroform–methanol (2:1, by volume) and applied to TLC-plates, which were developed in the freshly prepared organic phase of 2,2,4-trimethylpentane–ethyl acetate–acetic acid–water, 5:11:2:10 by volume [10].

Radioactive zones were located by radiochromatogram scanning (Berthold, LB 2760) and the standards visualized by spraying with 10% phosphomolybdic acid in ethanol and heating at 110° for 5 min. Zones corresponding to the different metabolites were scraped off and radioactivity (dpm) was determined by liquid scintillation counting with external standard quench correction. After subtraction of the radioactivity in the corresponding blank zone, and correction for the recovery (= total dpm removed from the TLC plate divided by dpm originally present in the aqueous mixture) the amount of product formed was calculated (pmole/mg tissue).

Measurement of endogenous prostacyclin biosynthesis. Peritoneal tissue (3–6 pieces, about 30 mg) was placed in 0.9 ml ice-cold 50 mM Tris buffer (pH 7.4 at 37°) to which endotoxin (0, 0.1 or 1.0 mg LPS in 0.1 ml 0.9% NaCl) was added at random; the reactions were started by placing the test-tubes in a shaking water bath at 37°. Incubations were terminated by cooling (0°), acidification (2 M citric acid) and tissue removal. Tritiated 6-oxo-$PGF_{1\alpha}$ (2000 dpm) was added as internal standard, and the mixture was extracted twice with 2 volumes of chloroform. The combined chloroform layers were dried under a stream of nitrogen and dissolved in 1 ml Tris buffer with 0.1% gelatin, and duplicate aliquots of 0.1 ml were used for the determination of tritium recovery (60–80%) and for the radioimmunoassay of 6-oxo-$PGF_{1\alpha}$. The results were expressed as pmole/mg.

For measurement of the net PGI_2 biosynthesis, aliquots (100 μl) of the incubation medium were removed both at zero time and after incubation at 37° for different periods. These aliquots were added to 0.1 ml ice-cold Tris buffer with 10 μg indomethacin, diluted with 0.8 ml Tris buffer and used for direct radioimmunoassay of 6-oxo-$PGF_{1\alpha}$. From the difference between both values the net prostacyclin biosynthesis was calculated and expressed as Δpmole/mg.

Radioimmunoassay of 6-oxo-$PGF_{1\alpha}$. Preparation of rabbit anti 6-oxo-$PGF_{1\alpha}$ antiserum [4] and radioimmunoassay of 6-oxo-$PGF_{1\alpha}$ were carried out as described by Beetens *et al.* [11]. The cross-reactivity of the antiserum with various prostaglandins was 1% for $PGF_{1\alpha}$ and less than 0.1% for PGE_2, 15-oxo-PGE_2, thromboxane B_2 and arachidonic acid [9].

Preparation of 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) [12, 13]. Briefly, AA (10 mg) was incubated twice at 0° with 1 mg soybean lipoxygenase in 0.1 M borate buffer, pH 9, for 30 min. After acidification (pH 4, 2 M citric acid) and double extraction with 100 ml ice-cold diethyl ether, the combined ether layers were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was dissolved in cold chloroform–methanol, and applied in one band on 2 mm precoated silica gel plates (Merck), which were developed in the solvent system described. The UV-absorbing zone with R_f value of 0.78 was eluted with diethyl ether–methanol (9:1) and stored. All procedures were carried out in the dark at 4°. The yield ranged between 41 and 44% assuming a molar extinction coefficient of 27,000.

Statistics. For all evalutions a 0.05 level of significance was chosen. When two groups of data were compared, Student's *t*-test was used. The one-way analysis of variance (ANOVA) was employed for the comparison of more than two groups of data. If the ANOVA indicated the existence of an overall treatment effect, the different means were compared with Duncan's new multiple range statistic [14].

RESULTS

The qualitative metabolism of a low concentration of exogenous AA by peritoneal tissue in Tris buffer is shown in Fig. 1. 6-Oxo-$PGF_{1\alpha}$ was the main metabolite, followed by smaller peaks co-chromatographing with 15-HETE, PGE_2 and $PGF_{2\alpha}$ (Fig. 1A). The 'polar shoulder' of the PGE_2 peak was sometimes observed and probably represents thromboxane B_2 (TXB_2). Unconverted AA was the only peak seen with boiled tissue (Fig. 1B). Indomethacin (28 μM) abolished the formation of PGE_2, $PGF_{2\alpha}$, TXB_2 and HHT, and strongly inhibited 6-oxo-$PGF_{1\alpha}$ biosynthesis (Fig. 1C). The formation of HETEs was not affected or slightly increased by indomethacin, whereas an additional inhibition of HETE biosynthesis was seen with 5 μM eicosatetraynoic acid (Fig. 1D).

The influence of exogenous substrate concentration on the formation of the main metabolites is shown in Fig. 2. The biosynthesis of cyclo-oxygenase products (6-oxo-$PGF_{1\alpha}$ > PGE_2 > $PGF_{2\alpha}$) reached a maximum at about 30 μM AA, whereas the formation of HETEs was saturated above 50 μM AA. The HETE curve was slightly sigmoid, and the rate of

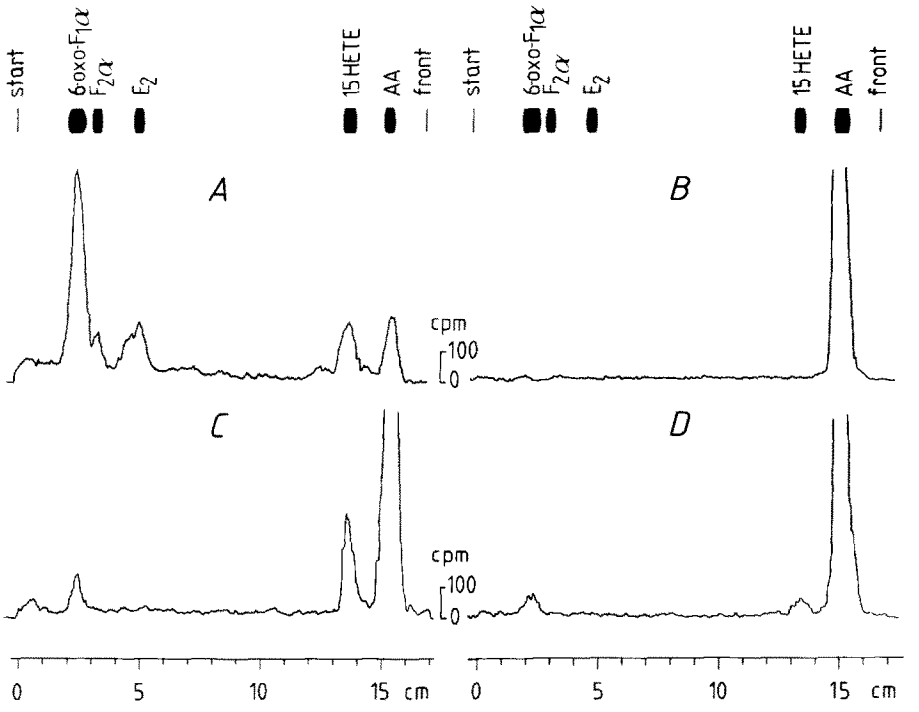

Fig. 1. Conversion of 0.8 μM arachidonic acid (AA) by rabbit peritoneal tissue (30 min, 37°). Products were extracted, separated by TLC and visualized by radiochromatogram scanning as described in Materials and Methods. A, control; B, blank, tissue preincubated at 100° for 5 min; C, tissue preincubated (5 min, 37°) and incubated with 28 μM indomethacin; D, tissue preincubated (5 min, 37°) and incubated with 5 μM eicosatetraynoic acid. The localization of the standards shown on top of the figure: 6-oxo-$F_{1\alpha}$ = 6-oxo-$PGF_{1\alpha}$; $F_{2\alpha}$ = $PGF_{2\alpha}$; E_2 = PGE_2; 15-HETE = 15-hydroxyeicosatetraenoic acid; AA = arachidonic acid.

HETE production exceeded the cyclo-oxygenase activity above 2.0 μM AA. This was easily seen when the ratio between HETEs and 6-oxo-$PGF_{1\alpha}$, the major cyclo-oxygenase product, was calculated (see Fig. 2).

In Fig. 3 the effect of the pH of the 50 mM Tris buffer on the conversion of 34.5 μM AA is shown. The pH optimum was 7.4 for the cyclo-oxygenase reaction, and 7.6 for formation of HETEs. In all further experiments a pH of 7.42 was used. As shown in Fig. 4 the reactions were completed within 40 min, and an incubation period of 30 min was used in the following experiments.

In this system the effects of endotoxin (0, 0.1 and

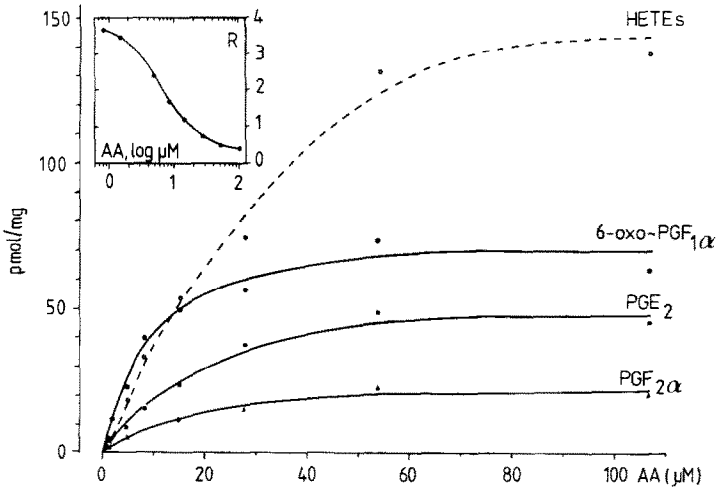

Fig. 2. Influence of arachidonic acid (AA) concentration on the formation of 6-oxo-$PGF_{1\alpha}$ (●), PGE_2 (■), $PGF_{2\alpha}$ (▲) and HETE (- - -) by rabbit peritoneum. The insert shows the ratio (R = 6-oxo-$PGF_{1\alpha}$/HETE) between 6-oxo-$PGF_{1\alpha}$ and HETEs at the different AA concentrations. Results are given as means of eight experiments.

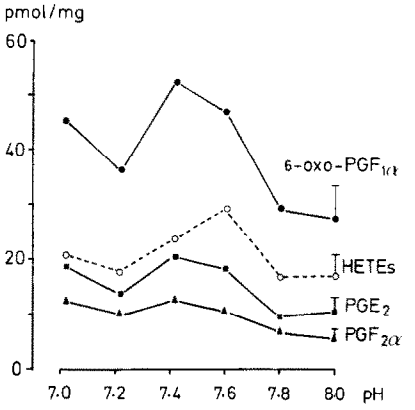

Fig. 3. Influence of pH conversion of 34.5 μM AA. Results are the mean of six experiments. For reasons of clarity the S.E.M. is only shown at pH 8.0.

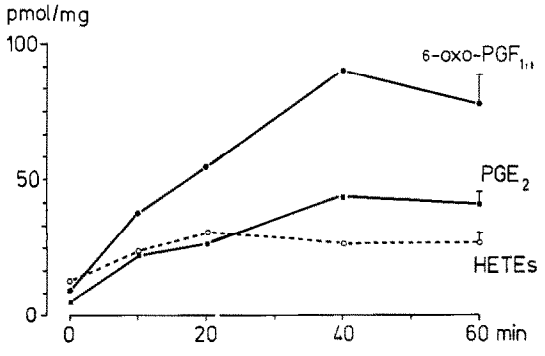

Fig. 4. Time curve of conversion of 34.5 μM AA. Results are given as means of four experiments. For reasons of clarity the S.E.M. is only shown at 60 min.

1.0 mg/ml) on the metabolism of AA were studied using two concentrations of exogenous AA, i.e. 0.8 and 34.5 μM (Fig. 5). At the lower substrate concentration, endotoxin inhibited the over-all biosynthesis of cyclo-oxygenase metabolites and 6-oxo-$PGF_{1\alpha}$ formation dose-dependently, whereas the inhibition of PGE_2 and $PGF_{2\alpha}$ was statistically significant at the higher endotoxin concentration.

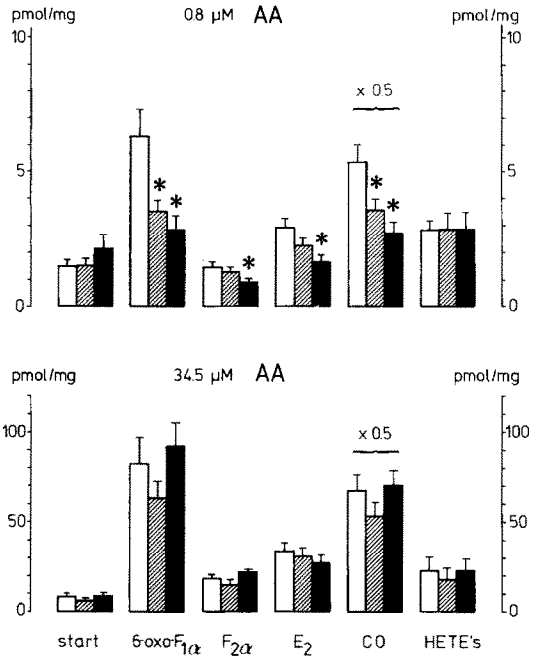

Fig. 5. Influence of endotoxin on the conversion of a low (0.8 μM, upper half) and high (34.5 μM, lower half) concentration of arachidonic acid. Rabbit peritoneal tissue was incubated for 30 min in 50 mM Tris, pH 7.42, with 0 (open columns), 0.1 (hatched columns) or 1.0 (closed columns) mg/ml LPS. 6-oxo-$F_{1\alpha}$ = 6-oxo-$PGF_{1\alpha}$, E_2 = PGE_2, $F_{2\alpha}$ = $PGF_{2\alpha}$, CO = total cyclo-oxygenase products (×0.5). *$P < 0.05$, Duncan.

Endotoxin did not influence the conversion of AA to HETEs or the amount of ^{14}C-label in the phospholipid zone. At the higher substrate concentration (34.5 μM AA), endotoxin did not change the biosynthesis of any of the metabolites studied.

In further experiments the endogenous PGI_2 biosynthesis by peritoneal tissue was assessed by radioimmunoassay of its non-enzymatic metabolite, 6-oxo-$PGF_{1\alpha}$. In the first series of incubations it was found that immunoreactive 6-oxo-$PGF_{1\alpha}$ was formed and 15-HPETE and indomethacin inhibited its biosynthesis, whereas 16.5 μM AA caused a two-fold stimulation (Table 1, experiment 1). In the presence

Table 1. Biosynthesis of 6-oxo-$PGF_{1\alpha}$ by isolated rabit peritoneum

Additions	Temp.	6-oxo-$PGF_{1\alpha}$ (pmole/mg in 20 min) Expt 1 ($n = 5$)	Expt 2 ($n = 8$)
— (blank)	0°	5.3 ± 1.0*	9.3 ± 0.9†
— (control)	37°	18.3 ± 1.6	18.1 ± 1.9
15-HPETE, 5 μM	37°	10.2 ± 1.4*	—
Indomethacin, 28 μM	37°	3.5 ± 0.4*	—
AA, 16.5 μM	37°	38.0 ± 3.0*	—
LPS, 0.1 mg/ml	37°	—	24.1 ± 2.2†
LPS, 1.0 mg/ml	37°	—	25.5 ± 1.8†

* ANOVA, $F(4, 20) = 36.23$, $P < 0.01$, Duncan's test ($\alpha = 0.05$) indicated that all means were statistically different from the control.

† ANOVA, $F(3, 28) = 17.26$, $P < 0.01$, all means statistically different from control (Duncan, $\alpha = 0.05$).

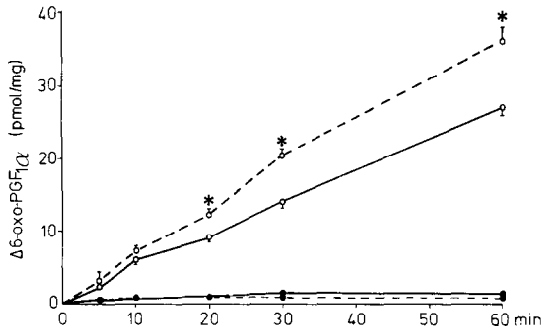

Fig. 6. Influence of endotoxin (0.1 mg/ml, broken lines) on the net endogenous production of prostacyclin (measured as immunoreactive 6-oxo-prostaglandin $F_{1\alpha}$, see Materials and Methods) in the presence (closed symbols) or absence (open symbols) of 28 μM indomethacin. Results given as mean ± S.E.M. for five incubations. *$P < 0.05$, Student's t-test, LPS-treated vs control.

of 0.1 and 1 mg endotoxin, the endogenous 6-oxo-$PGF_{1\alpha}$ biosynthesis was augmented (Table 1, experiment 2). In both series the blank values were relatively high (5–9 pmole/mg tissue after 30 min at 0°), strongly suggesting that tissue manipulation at the beginning and termination of the experiment caused significant prostacyclin biosynthesis. Therefore, aliquots (0.1 ml) for radioimmunoassay of 6-oxo-$PGF_{1\alpha}$ were taken from each incubation mixture at zero time, and after 5, 10, 20, 30 and 60 min at 37°; for each period of incubation the net 6-oxo-$PGF_{1\alpha}$ production was calculated by subtracting the value at 0 min. As shown in Fig. 6, rabbit peritoneal tissue formed a constant amount of 6-oxo-$PGF_{1\alpha}$ per unit of time. In the presence of 0.1 mg LPS/ml the rate of 6-oxo-$PGF_{1\alpha}$ formation was increased, leading to a statistically significant difference after 20 min. Indomethacin inhibited 6-oxo-$PGF_{1\alpha}$ biosynthesis in the presence or absence of LPS, and after 5 min about 75% inhibition was observed (see Fig. 5). This incomplete inhibition is in accordance with the partial suppression of the formation of exogenous PGI_2 observed after 5 min preincubation with indomethacin (Fig. 1). A contact period of 20–30 min was required for complete blockade of cyclo-oxygenase activity and the rate of endogenous PGI_2 formation was then zero.

DISCUSSION

Prostacyclin and C_{20}-monohydroxy fatty acids are the main metabolites of exogenous arachidonic acid in isolated rabbit peritoneal tissue, and the experiments with indomethacin and eicosatetraenoic acid confirmed the presence of cyclooxygenase, prostacyclin synthase and one or more lipoxygenases [6]. Cultured rabbit mesothelial cells convert exogenous arachidonate to PGI_2 and classical PGs (mainly PGE_2), whereas physicochemical analysis of their endogenous prostanoids revealed significant PGI_2 formation, but no detectable PGE_2 biosynthesis [8]. This suggests that mesothelial cells, the most abundant cell type in the tissue and present in much greater numbers than vascular endothelial cells, are the major source of PGI_2. Cultured mesothelial cells did not form hydroxy fatty acids from exogenous arachidonate [8], indicating that macrophages, leukocytes [15] or other cell types (fibroblasts, mast cells) were responsible for the lipoxygenase products. These consist predominantly of 13-HODE (from linoleic acid) and 15-HETE [9, 15].

Initially the optimum conditions for the formation of PGI_2 from exogenous AA were determined. Formation of cyclo-oxygenase products was saturated above 30 μM AA. The rate-limiting step in the overall conversion of exogenous AA to 6-oxo-$PGF_{1\alpha}$ by peritoneal tissue lies before the prostacyclin synthetase. Circumstantial evidence for this suggestion is the rather constant fraction of each cyclo-oxygenase product formed (e.g. at 28 μM AA: 6-oxo-$PGF_{1\alpha}$/PGE_2/$PGF_{2\alpha}$ = 0.57 ± 0.02/0.29 ± 0.02/0.14 ± 0.01) as compared to the much greater variation in the absolute amount (pmole/mg) of each cyclo-oxygenase product formed (e.g. at 28 μM AA: 6-oxo-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$, respectively 52 ± 9, 24 ± 3 and 14 ± 2).

Moreover, in contrast to ram seminal vesicle microsomes, in which the conversion of AA to PGH_2 exceeds PGI_2-synthase activity, and where a shift in favour of the non-enzymatic PGE_2 and $PGF_{2\alpha}$ is observed after reaching saturation of PGI_2 synthase activity with increasing AA concentrations [16], such a shift was not observed when AA exceeded the concentration at which 6-oxo-$PGF_{1\alpha}$ formation was maximal. Finally, when exogenous PGH_2 was used as substrate for PGI_2 synthase, relatively large quantities of 6-oxo-$PGF_{1\alpha}$ were formed (e.g. about 120 pmole 6-oxo-$PGF_{1\alpha}$/mg formed in 30 min with 30 μM PGH_2, [17]), despite the well known instability of PGH_2 in Tris buffer. Thus, either the penetration of AA to the cyclo-oxygenase or the cyclo-oxygenase activity as such are rate-limiting steps. The hypotonic buffer used in these studies probably favoured the access of AA to the site of conversion. The formation of 6-oxo-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ was reduced in isotonic buffers and, on the other hand, not increased after tissue disruption by repeated freezing and thawing (results not shown).

The influence of LPS (0.1 and 1 mg/ml) on the formation of 6-oxo-$PGF_{1\alpha}$ was studied using a limiting amount (0.8 μM) or an excess of exogenous AA. Under both conditions LPS did not affect 15-HETE formation or the incorporation of ^{14}C-label in the phospholipid fractions. However, LPS reduced the biosynthesis of radiolabelled cyclo-oxygenase products dose-dependently, but only at the lower concentration of AA. Since the inhibition was not seen at the higher AA concentration, it is unlikely that LPS inhibited cyclo-oxygenase activity or the preceding steps. Trapping of exogenous AA by LPS could also be excluded since uptake of exogenous AA by the tissue, the incorporation of ^{14}C-label in the phospholipid fraction, and the conversion of AA by the lipoxygenase pathway remained unchanged. A possible explanation could be that LPS caused the liberation of endogenous AA in the PGI_2-forming cells, resulting in isotope dilution and decreased formation of [1-^{14}C]-6-oxo-$PGF_{1\alpha}$.

The latter explanation is supported by measurements of endogenous prostacyclin biosynthesis, which was increased in the presence of LPS (Table 1, Fig. 5). This finding is in agreement with the stimulation of immunoreactive PGE formation by LPS in isolated rat mesentery [7]. However, in contrast to the peritoneal tissues, *in vitro* addition of LPS failed to stimulate the biosynthesis of PGE by the isolated rabbit jejunum [18], of PGI_2, PGE_2, TXB_2 and $PGF_{2\alpha}$ by rat lung strips [19], and of PGI_2 by the rat abdominal aorta and inferior vena cava [20], whereas an increased *ex vivo* biosynthesis of these prostanoids was observed when the same tissues were taken from LPS-treated animals [18–20]. The increased biosynthesis is probably explained by increased liberation of arachidonate, since a depression of cyclo-oxygenase activity has been observed in the kidney medulla of endotoxin-treated rats [21]. Based on the difference between the *ex vivo* and the *in vitro* experiments, Herman and Vane [18] suggested that activation of serum components, such as the complement system, was involved in the increased rate in prostanoid biosynthesis. Indeed, decomplementation suppressed the rise in arterial 6-oxo-$PGF_{1\alpha}$ levels in LPS-treated rabbits [22]. The abundant presence of macrophages, which are able to secrete complement components [23], could explain why *in vitro* addition of LPS stimulated the formation of prostanoids in rabbit (this study) and rat [7] peritoneal tissue, in contrast to the other tissues studied [18–20]. However, a direct stimulation of the AA release in the PGI_2-forming mesothelial cells cannot be excluded from our experiments.

Since high concentrations of endotoxin were required for induction of prostacyclin biosynthesis, it is unlikely that similar effects can explain the rise in arterial blood levels of 6-oxo-$PGF_{1\alpha}$ in endotoxin-treated rabbits [4, 22]. Therefore, the possibility that activated complement components are able to stimulate prostacyclin biosynthesis was investigated further, using the model of isolated peritoneal tissue. These experiments, in which activated serum complement enhances prostacyclin biosynthesis in this model, are described elsewhere [17].

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