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THE OCCURRENCE OF PROSTAGLANDIN E_2 IN SPLENIC VENOUS BLOOD OF THE DOG FOLLOWING SPLENIC NERVE STIMULATION

BY

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Prostaglandins are released when the sympathetic nerves supplying the rat epididymal fat pad *in vitro* are stimulated (Shaw, 1966). It is not yet known whether this is a general phenomenon associated with adrenergic nerve stimulation but the experiments described in this paper show that prostaglandin E_2 is released when the splenic nerve is stimulated. A preliminary account of this work has already been published (Davies, Horton & Withrington, 1967).

In the investigation reported here we collected venous blood samples from the perfused dog's spleen in order to detect any prostaglandin release when the splenic nerve was stimulated. A large increase in prostaglandin E_2 output was found.

METHODS

Blood-perfused dog spleen. An isolated spleen was perfused with blood from the femoral artery of a second (donor) dog using the technique described in the following paper (Davies & Withrington, 1968). The splenic venous blood was returned to the femoral vein of the donor except during the collection of a sample, when it was diverted out of a side arm. During the collection of a sample, blood from a reservoir was pumped into the donor. Electrodes were placed on the splenic nerve and the spleen was bathed in liquid paraffin at 37° C. The nerve was stimulated with 50 V pulses of 0.5 msec duration at 10/sec.

Extraction procedures. The procedures are outlined in Fig. 1. Blood was either collected in a measuring cylinder and added to 4 vol. ice cold 96% aqueous ethanol, or added to the ethanol directly from the collecting cannula. The mixture was shaken and filtered. The residue was re-extracted with 4 vol. 96% aqueous ethanol. The combined aqueous ethanol filtrates were evaporated to dryness. The dried residue was dissolved in a mixture of equal volumes of ethyl acetate and water at pH 3. After separation, the acid acqueous phase was re-extracted with 1 vol. ethyl acetate. The combined ethyl acetate phases were concentrated to smaller volume and extracted twice with an equal volume of phosphate buffer solution pH 8. The aqueous phases were pooled, acidified to pH 3 by addition of HCl, and re-extracted twice with an equal volume of ethyl acetate. The ethyl acetate phases were pooled and evaporated to dryness.

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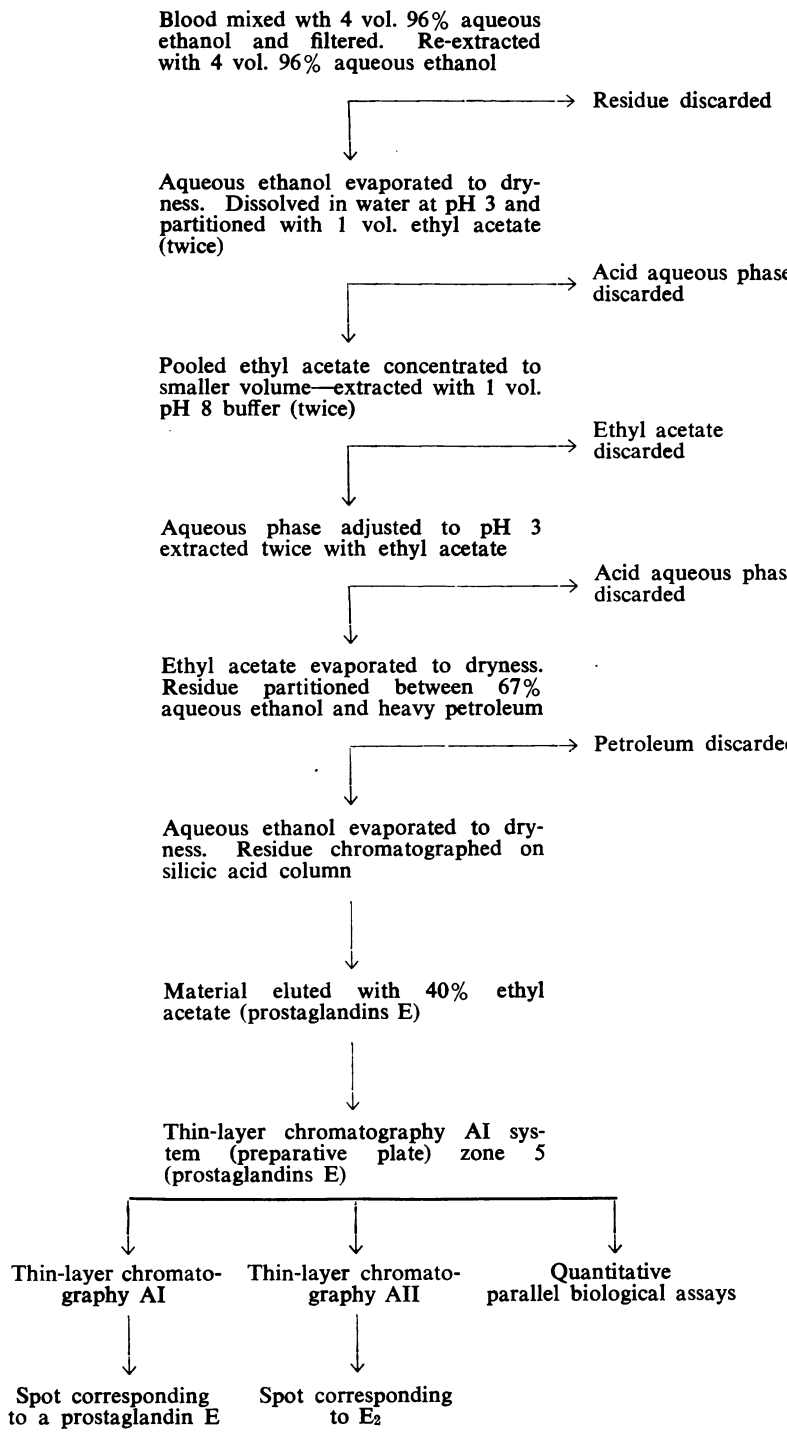

Fig. 1. Extraction and identification of prostaglandins.

The residue was partitioned between equal volumes of 67% aqueous ethanol and heavy petroleum (b.p. 60°–80° C). The aqueous ethanol was evaporated to dryness and chromatographed on silicic acid columns (1–3 g) using the procedure described by Horton & Main (1967). Thin-layer plates of dimensions 200 × 50 mm or 200 × 200 mm coated with Silica Gel G (E. Merck) 0.25 mm thick were prepared and used as previously described in detail (Horton & Main, 1967). The AI and AII solvent systems were described by Gréen & Samuelsson (1964).

Biological assays. The rat fundus was used routinely for the estimation of biological activity in the various fractions obtained during the extraction procedure. With all smooth muscle preparations contractions were recorded on a smoked drum using a frontal writing lever.

Rat fundus. A fundal strip was prepared and suspended in a 10 ml. bath containing oxygenated Tyrode solution. Doses were in contact with the tissue for 1.5–2 min and a cycle of 6–8 min was used.

Rabbit jejunum. Proximal jejunum, from rabbits weighing 1–3 kg, was suspended in a 4 ml. bath containing Tyrode solution at 37° C and gassed with air. A dose cycle of 4–5 min with 45–90 sec contact was used.

Guinea-pig ileum. Terminal ileum from guinea-pigs weighing 150–600 g was suspended in a 4 ml. bath containing Tyrode solution at 37° 7 and gassed with air. A dose cycle of 5 min with 1 min contact was used.

Cat tracheal chain. A chain of three tracheal segments was suspended in Krebs-Henseleit solution at 37° C and gassed with 5% CO_2–95% O_2. Contractions were elicited by adding acetylcholine to the bath and the assay was performed by comparing the inhibitory action of the unknown extract with that of prostaglandin E_2 (Horton & Main, 1967).

RESULTS

Occurrence of prostaglandin-like substances in splenic venous blood

In a preliminary experiment samples of plasma were acidified with HCl and partitioned with an equal volume of ethyl acetate. After two such extractions, the combined ethyl acetate phases were evaporated to dryness. The residue was dissolved in water and assayed on the rat fundus.

The sample collected during and after electrical stimulation of the splenic nerves (300 stimuli at 10/sec) contained the equivalent of 35 ng prostaglandin E_1/ml. plasma, whereas the control samples did not contain any detectable prostaglandin. After phenoxybenzamine, administered in a dose (5 mg) sufficient to block the contraction of the spleen to nerve stimulation, the output of prostaglandin-like material in response to nerve stimulation was abolished, whereas the output of noradrenaline was increased (Table 1).

TABLE 1

PLASMA CONCENTRATION OF PROSTAGLANDIN-LIKE SUBSTANCES IN SPLENIC VENOUS BLOOD OF A DOG EXPRESSED AS PROSTAGLANDIN E_1 EQUIVALENT

	Prostaglandin E_1 (ng/ml.)	Noradrenaline (ng/stimulus)
Before phenoxybenzamine		
Venous control	<2	
Nerve stimulation (300 at 10/sec)	35	1·02
After phenoxybenzamine 5 mg		
Venous control	<6	
Nerve stimulation (300 at 10/sec)	<6	5·87

A similar increase in prostaglandin-like activity was found in four out of five dogs investigated. In a sixth dog sufficient prostaglandin E_2 for identification was extracted from the blood collected during stimulation.

Identification of prostaglandin E_2

Splenic venous blood (46 ml.) collected during and after nerve stimulation was extracted by the solvent partition procedure outlined in Fig. 1. The material was chromatographed on a silicic acid column and the eluates were assayed for biological activity on the isolated rat fundus. Several peaks of activity were found (Fig. 2), the one eluted with 40% ethyl acetate corresponding to the prostaglandins E as found in other experiments (Horton & Main, 1967 ; Holmes & Horton, 1967). The material from the " prostaglandin E " peak was purified further by thin-layer chromatography on silica gel plates using the AI solvent system of Greén & Samuelsson (1964). About one-third of the biological activity of this peak was found in the zone corresponding to the R_F value of prostaglandin E_1. The remaining two-thirds had a higher R_F value (see below).

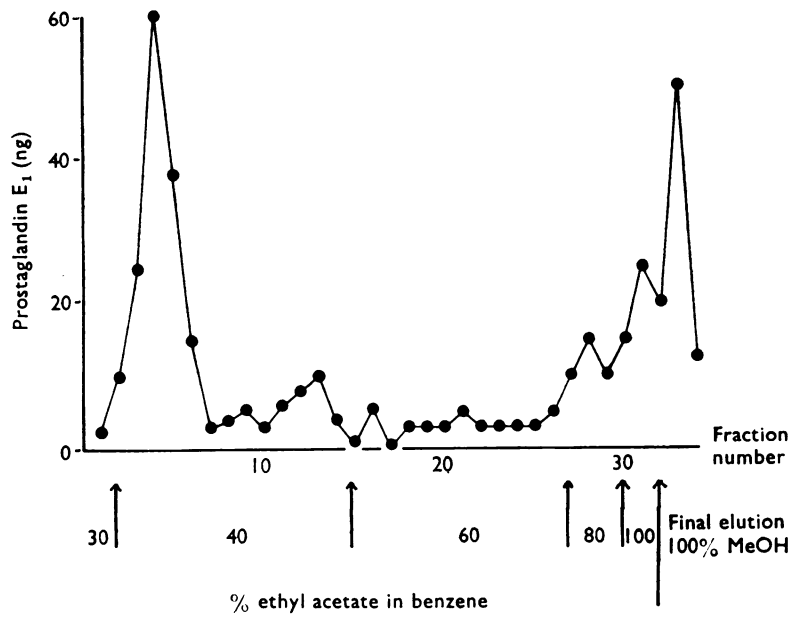

Fig. 2. Silicic acid chromatography (1 g column) of splenic blood extracts. Elution at 1 ml./min ; 20 ml. fractions collected. Ordinate: biological activity assayed on the rat fundus in terms of ng of prostaglandin E_1. Abscissa: upper line, fraction number ; lower line, % of ethyl acetate in benzene used for elution.

These results were confirmed with a larger sample (252 ml.) of blood collected from another dog during 60 sec of splenic nerve stimulation. In this experiment an activity equivalent to 20 μg of prostaglandin E_1 was eluted with 40% ethyl acetate in benzene. The material from this peak was run on a preparative thin-layer plate in the AI solvent system, the zone corresponding to the prostaglandins E was eluted and divided in three aliquots. One was re-chromatographed in the AI system. On spraying the plate with phosphomolybdic acid only one spot was seen corresponding to the R_F value (0.54) of the prostaglandins E. The second aliquot was chromatographed on a silica gel plate impregnated with $AgNO_3$ in the AII solvent system. On spraying with phosphomolybdic

acid only one spot appeared, corresponding to the position of prostaglandin E_2 (R_F 0.53). On the marker plate prostaglandins E_2 and E_1 had mean R_F values of 0.52 and 0.74 respectively.

The third aliquot from the AI preparative plate was used for parallel biological assays using prostaglandin E_2 as the standard for comparison (Table 2). Like prostaglandin E_2 the prostaglandin extracted from splenic blood contracted the rat fundus, guinea-pig ileum and rabbit jejunum but inhibited acetylcholine contractions of the cat trachea. The assay results on the four tissues also agreed well quantitatively.

TABLE 2

PARALLEL BIOLOGICAL ASSAYS OF A FINAL EXTRACT OF SPLENIC VENOUS BLOOD IN TERMS OF PROSTAGLANDIN E_2

Rat fundus	0·6 (0·4–0·8)
Guinea-pig ileum	0·6 (0·4–0·8)
Rabbit jejunum	0·7 (0·5–1·0)
Cat trachea	0·6 (0·2–1·0)

Occurrence of other lipids in extracts of splenic venous blood

In addition to prostaglandin E_2 several other biologically active lipids were found in extracts of splenic venous blood collected during and after splenic nerve stimulation. On thin-layer chromatography in the AI solvent system of the material in the "prostaglandin E" peak from the silicic acid column (Fig. 2), four substances with an R_F value greater than the prostaglandins E were detected in addition to the prostaglandin E_2. A mixture containing these four substances was biologically active on the rat fundus, but they have not been studied individually.

When the material in fractions 27–33 from the silicic acid column (Fig. 2) was run on thin-layer plates, at least two spots were present in each fraction as visualized by spraying with phosphomolybdic acid. We have no evidence as to which of these contained the biologically active principle.

In one experiment a small amount of prostaglandin F was also detected. The evidence for this was based on its chromatographic behaviour on column and thin-layer chromatography and on parallel biological assays.

Free fatty acid levels

In several experiments unesterified fatty acid levels were estimated by titrating an aliquot of the material before partitioning between petroleum and 67% ethanol. The residue was dissolved in ethanol and titrated with N/100 alcoholic KOH using thymol blue as the indicator. An experiment (Fig. 3) illustrates the increase in prostaglandin E_2 and a slight decrease in free fatty acid levels in the sample collected during and after nerve stimulation. This confirms that the nerves to adipose tissue adjacent to the spleen were not being stimulated.

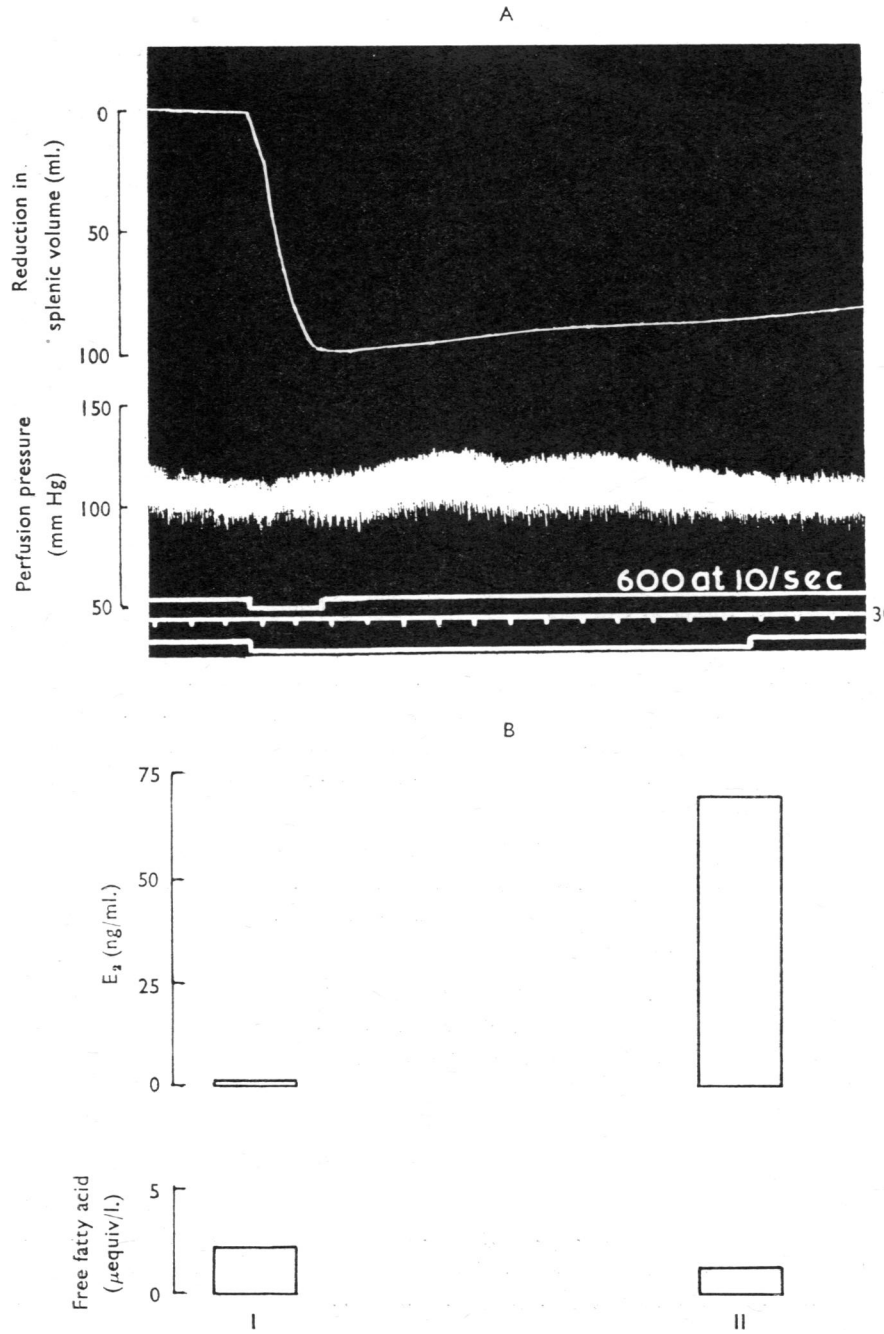

Fig. 3. Dog, 11.0 kg. A: Reduction in splenic volume in response to a train of 600 stimuli at 10/sec applied to the splenic nerve. A pooled blood sample was collected for the 7 min period during and after the stimulus, as indicated by the event marker. B: Assay of prostaglandin E_2 and free fatty acids in a control blood sample I taken 3 min before nerve stimulation and the blood sample II.

Identification of a prostaglandin E in extracts of dog spleen

Five dog spleens weighing a total of approximately 400 g were macerated in ethanol and extracted by the procedure outlined in Fig. 1. On the basis of column and thin-layer chromatography it was concluded that dog spleen contains a prostaglandin E, but there was insufficient material for identification by chromatography in the AII solvent system. On the basis of extracting another spleen weighing 24 g the concentration seemed to be of the order of 2 ng prostaglandin E_2/g tissue.

DISCUSSION

Identification of prostaglandin E_2

As discussed at length by Horton & Main (1967), the identification of prostaglandins on the basis of chromatographic behaviour and biological activity is not conclusive but the evidence is strongly suggestive. From the experiments described in this paper it can be stated that the behaviour of the substance isolated from splenic venous blood collected during and after nerve stimulation was identical to prostaglandin E_2 on three chromatographic systems and could not be distinguished from prostaglandin E_2 by quantitative parallel biological assay on four tissues, three of which contracted and one of which relaxed in response to prostaglandin E_2. It is certain that this activity was not caused by a prostaglandin F nor by prostaglandins E_1 or E_3. Nor from the data published by others was it likely to have been prostaglandin A_1, B_1, A_2 or B_2 or one of their 19-hydroxy-derivatives (Hamberg & Samuelsson, 1966).

Presence of other prostaglandin-like substances

Although the major biologically active constituent of the extracts was prostaglandin E_2, the presence of other prostaglandins cannot be excluded. There were certainly other substances with partition and chromatographic behaviour similar to the prostaglandins, but the nature of these is unknown except in one experiment in which a prostaglandin F was tentatively identified. It seems likely that the stimulus which releases prostaglandin E_2 causes a simultaneous release of several prostaglandin-like substances. This fact should be taken into account when considering the physiological implications of these observations.

Origin of the prostaglandins

The absence of any increase in free fatty acid levels when splenic nerves were stimulated excludes the possibility that the prostaglandins arise from adipose tissue adjacent to the spleen. This is not to say that fatty acids other than prostaglandins are not released from the spleen itself, but the amounts concerned are likely to be minute compared with the large output which occurs when fat is mobilized.

The absence of prostaglandins in samples collected after administration of phenoxybenzamine suggests that the prostaglandins are released in response to the action of noradrenaline at the post-synaptic site but an action of phenoxybenzamine in blocking prostaglandin release from a pre-synaptic site, though less likely, cannot be excluded.

Physiological significance of prostaglandin release

Prostaglandins are kidely distributed in animal tissues (Bergström & Samuelsson, 1965) and are known to be released in response to a variety of stimuli. For example they are released from the central nervous system on sensory nerve stimulation (Ramwell & Shaw, 1966), from the rat isolated diaphragm on stimulation of the phrenic nerve (Ramwell, Shaw & Kucharski, 1965), from the adrenal glands in response to acetylcholine (Ramwell, Shaw, Douglas & Poisner, 1965) and from adipose tissue in response to adrenergic nerve stimulation (Shaw, 1966). The experiments in this paper provide a second instance of prostaglandin release on stimulation of adrenergic nerves, although there is some evidence that the release from the diaphragm is caused by stimulation of adrenergic fibres in the phrenic nerve (Ramwell *et al.*, 1965). Our experiments provide the first demonstration that prostaglandins are released directly into the blood from an organ. It is significant that the output following nerve stimulation for 1 min was greatly in excess of the total amount of prostaglandin which could be extracted from a spleen. This suggests a rapid synthesis and agrees with the findings on cat adrenal glands (Ramwell *et al.*, 1965).

In view of the known inhibitory actions of prostaglandins E on the adenyl cyclase system which, in many instances, is thought to mediate the actions of adrenaline and noradrenaline, the possibility of a local feedback mechanism has been envisaged by various workers (see Bergström & Samuelsson, 1967). Prostaglandin E_1 antagonizes some of the vascular effects of the catecholamines (Holmes, Horton & Main, 1963), but the results in the following paper indicate that such an antagonism does not occur in the dog spleen (Davies & Withrington, 1968). Further information is needed about the mechanism of prostaglandin release and about release from other organs on sympathetic nerve stimulation, before any conclusions can be drawn as to how these findings fit into the picture of events at adrenergic synapses.

SUMMARY

1. In five dogs prostaglandin-like activity was detected in splenic venous blood samples collected during and after nerve stimulation.

2. Prostaglandin E_2 was identified in extracts of the splenic blood by its behaviour on solvent partition, chromatography in three systems, and by its biological activity on four isolated tissues.

3. Several other lipids which stimulate smooth muscle were also detected but these have not been identified.

4. Prostaglandin output was blocked by the administration of phenoxybenzamine (5 mg), whereas noradrenaline output was increased.

5. Splenic nerve stimulation did not increase free fatty acid levels in splenic venous blood, which excludes the possibility that the prostaglandins arise from adipose tissue adjacent to the spleen.

6. A prostaglandin E has been extracted from splenic tissue but the amounts present were too small to permit complete identification.

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