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# Effect of phenobarbitone pretreatment upon endothelium-dependent relaxation to acetylcholine in rat superior mesenteric arterial bed

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**1** Pretreatment of rats for 5 days with phenobarbitone (80 mg $kg^{-1}$ $day^{-1}$) enhanced the potency of acetylcholine in opposing noradrenaline-induced vasoconstriction in the isolated perfused superior mesenteric arterial bed; in 10 saline-pretreated control animals the $ED_{50}$ was 14.0 ± 3.9 ng whereas it was 3.23 ± 1.00 ng in 10 phenobarbitone-pretreated animals.

**2** In both saline- and phenobarbitone-pretreated rats acetylcholine was ineffective at opposing noradrenaline vasoconstriction after the mesentery had been perfused for 90 s with a 0.3% solution of the detergent CHAPS in distilled water (to remove the endothelium), but pressor responses to noradrenaline were unaffected.

**3** Pretreatment with phenobarbitone had no effect on the opposition by sodium nitroprusside of noradrenaline pressor responses. Also, the effects of nitroprusside were not affected by perfusion with CHAPS in either control or barbiturate-pretreated groups.

**4** Inclusion of indomethacin (10 $\mu$M) in the perfusion fluid had no effect on the enhancement by phenobarbitone pretreatment of the endothelium-dependent opposition by acetylcholine of noradrenaline pressor responses; the $ED_{50}$ values in the absence and presence of indomethacin were, respectively, 2.40 ± 0.31 ng and 1.87 ± 0.27 ng ($n = 6$).

**5** The concentration of cytochrome P450 in the microsomal fraction obtained from the mesenteric preparation was increased from 204 ± 32 (saline-pretreated; $n = 7$) to 784 ± 249 pmol $g^{-1}$ wet wt ($n = 7$) by the phenobarbitone pretreatment.

**6** It is concluded that the increase in potency of acetylcholine as an endothelium-dependent vasodilator by phenobarbitone pretreatment is most probably at the level of the endothelium rather than the vascular smooth muscle.

## Introduction

Vascular relaxation in response to many vasodilator agents is now known to be due to the mediation of the endothelium, which produces and releases a humoral factor in response to the applied agonists. It is this agent, termed endothelium-derived relaxing factor (EDRF), which brings about the relaxation of the underlying vascular smooth muscle (Furchgott & Zawadzki, 1980; Furchgott, 1983). Recently it has been shown that many of the properties of EDRF are shared by nitric oxide and this has led to the suggestion that EDRF might be identical with this nitrogen free radical (Furchgott *et al.*, 1987; Ignarro *et al.*, 1987; Palmer *et al.*, 1987). However, on the basis of the differential sensitivity of various smooth muscles to nitric oxide and EDRF, Shikano *et al.* (1987) have cast doubt on this simple identity.

It is therefore possible that there might be more than one factor involved in endothelium-dependent relaxation, especially since some species-dependent differences have been demonstrated (Forstermann & Neufang, 1984b). Early work had suggested that arachidonic acid metabolism might be involved in endothelium-dependent relaxation since mepacrine, a phospholipase $A_2$ inhibitor, and eicosatetraynoic acid, an analogue inhibitor of arachidonic acid metabolism, inhibited endothelium-dependent relaxation to acetylcholine (Furchgott & Zawadzki, 1980; Zawadzki *et al.*, 1980). However, neither the cyclooxygenase inhibitor, indomethacin, nor the dual

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cyclo-oxygenase and lipoxygenase inhibitor, BW755C, affected this process (Chand & Altura, 1981; Spokas & Folco, 1984). Thus attention turned to the metabolism of arachidonic acid by cytochrome P450-dependent mono-oxygenase, the 'epoxygenase pathway' (Morrison & Pascoe, 1981; Capdevila *et al.*, 1981). In apparent support of this, SKF-525A, an inhibitor of microsomal cytochrome P450, was shown to interfere with agonist-induced endothelium-dependent relaxation (Singer *et al.*, 1984; Pinto *et al.*, 1986; 1987). However, bioassay experiments in which EDRF was placed in contact with putative inhibitors after production but before bioassay showed that SKF-525A interacted with the factor in solution, rather than interfered with its production or action at the recipient tissue (Förstermann *et al.*, 1988). Förstermann and his coworkers, though, have evidence from the use of thimerosal, an inhibitor of acyl-coenzyme A: lysolecithin transferase activity in endothelial cells, that fatty acid metabolism may be involved in EDRF production (Förstermann & Neufang, 1984a; Föstermann *et al.*, 1986a,b). It is noteworthy that cytochrome P450 has been detected in vascular tissue (Juchau *et al.*, 1976) and has been localized in the endothelium (Abraham *et al.*, 1985; Pinto *et al.*, 1986). The products of cytochrome P450 mono-oxygenase-dependent metabolism of arachidonic acid include biologically active substances amongst which are epoxides and diols (Capdevila *et al.*, 1981); the 5,6-epoxide of arachidonic acid has been shown to be a vasodilator (Carroll *et al.*, 1987; Proctor *et al.*, 1987).

Pretreatment of rats with phenobarbitone, a potent inducer of hepatic cytochrome P450, is known to cause an increase in liver blood flow (Ohnhaus *et al.*, 1971; Yates *et al.*, 1978) and this has been shown to be accompanied by a decrease in resistance to flow through the superior mesenteric arterial bed *in situ* (Hiley *et al.*, 1985). It was therefore of interest to examine the influence of phenobarbitone pretreatment upon endothelium-dependent relaxation in the superior mesenteric arterial bed of the rat.

## Methods

### *Pretreatment of animals*

Male Wistar rats (280–300 g; Bantin & Kingman, Hull, Humberside) were injected twice daily for 5 days with 40 mg $kg^{-1}$ phenobarbitone (BDH, Poole, Dorset) in a volume of 2 ml $kg^{-1}$ isotonic saline. Control rats received the same volume of 0.9% saline twice daily for 5 days. Experiments were carried out on the sixth day after the start of treatment.

### *Perfusion studies*

The animals were anaesthetized with sodium pentobarbitone (60 mg $kg^{-1}$, i.p.: Sagatal; May & Baker, Dagenham, Essex), heparinized (1000 u $kg^{-1}$, i.p.), the superior mesenteric artery cannulated and the associated vascular bed perfused, using a Harvard Type 1203A perfusion pump, according to the method of MacGregor (1965), at 2 ml $min^{-1}$ with Krebs-Henseleit solution containing (mM): NaCl 118, KCl 4.7, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and D-glucose 5.5. The perfusing solution was saturated with 5% $CO_2$/95% $O_2$ and, together with the tissue, maintained at 37°C. Perfusion pressure was recorded by a Bell & Howell Type 4-422-0001 pressure transducer connected to a T-piece placed in the perfusion circuit close to the point of insertion into the superior mesenteric artery. Recordings were made on a Grass Model 79D polygraph.

After a 30 min equilibration period, drugs were administered into the perfusion circuit in volumes of 200 $\mu$l or less. The pressor response to a submaximal dose of noradrenaline (10 $\mu$g in 100 $\mu$l; Koch-Light, Haverhill, Suffolk) was determined every 2 min. The vasorelaxant effects of acetylcholine (Sigma) were determined by co-administration of various doses as a bolus with the noradrenaline test dose (Hiley *et al.*, 1987).

Endothelium destruction was achieved by perfusion of the vascular bed with a 0.3% (w/v) solution of the detergent CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate; Sigma Chemical Co., Poole, Dorset) followed by 45 min re-equilibration. This procedure abolishes the relaxant response to acetylcholine and histological examination has shown that the endothelium is removed from the vessels (Hiley *et al.*, 1987).

### *Cytochrome P450 determinations*

Pretreated animals were killed by a blow to the back of the head and the mesentery removed. This was rinsed in ice-cold buffer (1.15% (w/v) KCl and 50 mM phosphate, pH 7.4), in order to remove residual blood, before being homogenized in 4 vol. ice-cold KCl/phosphate buffer. The homogenate was centrifuged at 10,000 *g* for 30 min. The resulting supernatant was retained and the pellet was washed in another 4 vol. KCl/phosphate buffer and centrifuged again at 10,000 *g* for 30 min. The combined supernatants were then centrifuged at 100,000 *g* for 60 min; all centrifugations were carried out at 4°C. The microsomal pellet was resuspended in 3 ml ice-

cold KCl/phosphate buffer. The protein concentration was measured by the method of Lowry *et al.* (1951) and the amount of cytochrome P450 determined by the method of Omura & Sato (1964).

*Data analysis and statistics*

Dose-response relationships were analysed by fitting a logistic equation:

$$R = \frac{R_{max} \cdot A^n}{ED_{50}^n + A^n}$$

where R is the reduction of the noradrenaline response by acetylcholine, A the acetylcholine concentration, $R_{max}$ the maximal response and $n$ a slope function. A modified Marquardt procedure was used as described by Aceves *et al.* (1985).

Statistical comparisons were made by Student's $t$ test and values are given as mean ± s.e.mean; $n$ represents the number of animals in a group.

## Results

The pressor response to 10 μg noradrenaline was the same in both phenobarbitone and control groups being, respectively, 50.1 ± 2.9 ($n = 10$) and 50.3 ± 4.6 mmHg ($n = 10$). This response was not significantly affected after perfusion with 0.3% CHAPS (52.2 ± 6.0 mmHg, phenobarbitone; 45.0 ± 6.3 mmHg, control).

Figure 1 shows that in phenobarbitone-pretreated rats there was a significant, 4.3 ± 2.6 fold, parallel leftward shift of the log-dose response curve to acetylcholine relative to the controls. The calculated $ED_{50}$ values were significantly different ($P < 0.05$) being 3.2 ± 1.0 ng (phenobarbitone) and 14.0 ± 3.9 ng (saline). The slope functions were unchanged at 0.58 ± 0.01 and 0.53 ± 0.05, respectively, in phenobarbitone and control rats, as were the maximum reductions in the noradrenaline responses by acetylcholine (75.7 ± 3.9%, control; 71.3 ± 4.6%, phenobarbitone). CHAPS perfusion abolished the inhibition of the noradrenaline pressor response by acetylcholine in both groups (Figure 1).

The phenobarbitone pretreatment had no significant effect on the opposition to the noradrenaline pressor response by sodium nitroprusside (Figure 2a and b), nor did the removal of the endothelium with CHAPS have any effect on these responses. In the

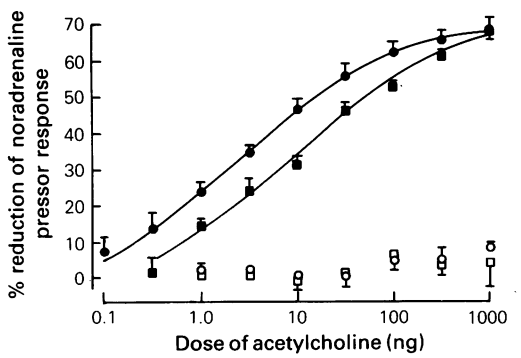


**Figure 1** Reduction of the pressor response to 10 μg noradrenaline by co-administered acetylcholine in isolated, perfused superior mesenteric arterial beds taken from rats pretreated with either phenobarbitone (●, ○; $n = 10$) or saline (■, □; $n = 10$). (●, ■) Represent data obtained before, and (○, □) results obtained after, perfusion of the mesentery with CHAPS. The lines drawn are those obtained from the non-linear least squares fitting procedure and values are shown as the mean with the vertical lines representing s.e.mean.

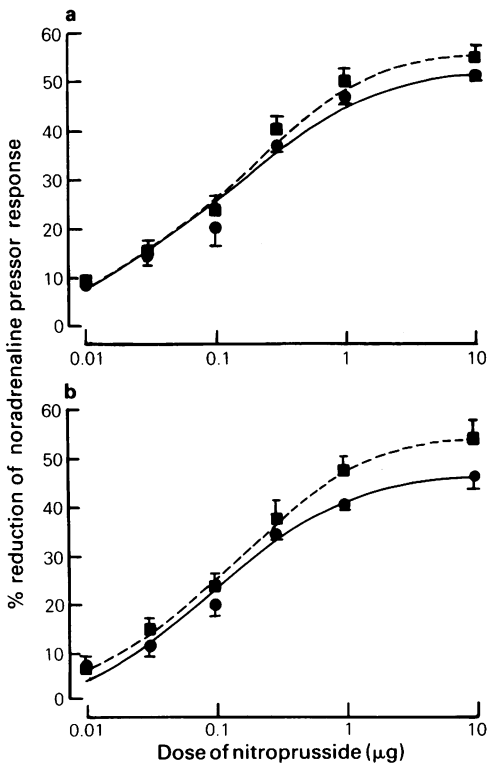


**Figure 2** Opposition to the pressor response to 10 μg noradrenaline by co-administered sodium nitroprusside in isolated, perfused superior mesenteric arterial beds taken from rats pretreated with either (a) saline ($n = 10$) or (b) phenobarbitone ($n = 10$). (●——●) Data obtained before, and (■- - -■) after perfusion of the mesentery with CHAPS. The lines drawn are those obtained from the non-linear least squares fitting procedure. The values shown are the mean with the vertical lines representing s.e.mean.

presence of the endothelium, the calculated maximum values for the inhibition of the noradrenaline pressor response were 54.2 ± 2.6% and 46.5 ± 4.0%, respectively, for the control and phenobarbitone-pretreated animals ($n = 10$ for both groups). After perfusion with CHAPS these values were 57.4 ± 3.7% (control) and 57.0 ± 2.7% (phenobarbitone). The $ED_{50}$ values and slope functions ($n$) were, respectively, 112 ± 29 ng and 0.74 ± 0.08 (control with endothelium); 112 ± 34 ng and 0.88 ± 0.19 (phenobarbitone with endothelium); 113 ± 30 ng and 0.75 ± 0.13 (control after CHAPS) and 139 ± 27 ng and 0.74 ± 0.07 (phenobarbitone after CHAPS).

In another 6 animals pretreated with phenobarbitone, log dose-response relationships were obtained for the opposition to the noradrenaline pressor response by acetylcholine; the calculated values for $ED_{50}$ and maximum response were 2.40 ± 0.31 ng and 69.8 ± 1.9%, respectively. During perfusion with 10 $\mu$M indomethacin, the values were 1.87 ± 0.27 ng and 71.8 ± 2.2%. There was no statistical difference between either of these two pairs of values.

The liver was removed from each animal used in these perfusion experiments and weighed. The mean weights of the livers removed from saline and phenobarbitone-pretreated animals were, respectively, 3.45 ± 0.14 ($n = 20$) and 4.49 ± 0.16 g 100 $g^{-1}$ body wt ($n = 25$). The livers from the phenobarbitone group were significantly heavier than those from the control animals by 30.1% ($P < 0.001$). A similar increase of 27.2% in liver size was found in the rats used in the experiments to determine mesenteric cytochrome P450 content, where the mean sizes were 4.61 ± 0.20 (saline; $n = 7$) and 5.86 ± 0.21 g 100 $g^{-1}$ body wt (phenobarbitone; $n = 7$). Table 1 shows that there was a 286% greater content of this cytochrome in the mesenteries from rats pretreated with the barbiturate, although there was no difference in the sizes of the mesenteries removed from the two groups of rats. This increase in cytochrome P450 content was almost entirely due to a 180% increase in microsomal protein; there was no significant difference between the saline and phenobarbitone groups with respect to the ratio of cytochrome P450 to microsomal protein (Table 1).

## Discussion

The superior mesenteric bed of the rat has been shown previously to dilate in response to acetylcholine in an endothelium-dependent manner (Burdet *et al.*, 1986; Byfield *et al.*, 1986; Hiley *et al.*, 1987). The present study shows clearly that pretreatment with phenobarbitone increases the sensitivity of the superior mesenteric arterial bed to the vasorelaxant activity of acetylcholine. The maximum effect of acetylcholine was not, however, changed by the pretreatment. Perfusion of the mesentery with CHAPS, a zwitterionic detergent, in distilled water for 90 s abolished the relaxant effect of acetylcholine but left the response to noradrenaline unaltered. Previous work has shown that perfusion with detergent under appropriate conditions removes the endothelium from the blood vessels of the mesentery (Burdet *et al.*, 1986; Byfield *et al.*, 1986; Hiley *et al.*, 1987) and, functionally, this may be confirmed by showing that acetylcholine no longer has an effect as a relaxant. It is interesting to note that, unlike larger arteries such as the aorta or coronary arteries (Furchgott & Zawadzki, 1980), acetylcholine has no spasmogenic effect in this vascular bed after removal of the endothelium.

Although pretreatment with phenobarbitone increased the effectiveness of acetylcholine as an endothelium-dependent vasorelaxant, it is possible that it is the sensitivity of the smooth muscle, rather than the activity of the endothelium that has been affected. Therefore we investigated the effects of the pretreatment on the responses to nitroprusside, which brings about vasorelaxation by generating nitric oxide in the smooth muscle cell (Ignarro *et al.*, 1981; Bennet & Marks, 1984). The release of nitric

**Table 1** Effect of pretreatment of rats with phenobarbitone (80 mg $kg^{-1}$ $day^{-1}$) on mesenteric microsomal protein and cytochrome P450 contents

| | *Saline* ($n = 7$) | *Phenobarbitone* ($n = 7$) |
|---|---|---|
| Mesenteric weight (g 100 $g^{-1}$ body wt) | 0.78 ± 0.05 | 0.79 ± 0.03 |
| Microsomal protein (mg $g^{-1}$ mesentery) | 2.18 ± 0.34 | 6.10 ± 0.96** |
| Microsomal cytochrome P450 (nmol $g^{-1}$ mesentery) | 0.204 ± 0.032 | 0.784 ± 0.249* |
| (nmol $mg^{-1}$ protein) | 0.109 ± 0.026 | 0.154 ± 0.052 |

Statistical significance between the saline control and phenobarbitone pretreated groups was assessed by Student's $t$ test: *$P < 0.05$; **$P < 0.01$.

oxide from nitrovasodilators takes place within the smooth muscle and the generated oxide stimulates the soluble guanylyl cyclase within the sarcoplasm (Gruetter *et al.*, 1981); the process is therefore endothelium-independent. Our data show that perfusion with CHAPS had no effect upon the opposition to the noradrenaline pressor response by sodium nitroprusside, confirming the endothelium-independent nature of the response. We also found that the pretreatment with phenobarbitone had no effect on the vasorelaxation to nitroprusside and, thus, was not affecting the smooth muscle directly. The change in the sensitivity of the tissue to acetylcholine must therefore be at the level of the endothelium.

It is interesting to note that the maximum effect of acetylcholine as a vasodilator (a reduction of over 70% in the noradrenaline pressor response) was significantly greater than that of nitroprusside (a maximum reduction of 57%). Since the nitrovasodilators are thought to work by the intracellular generation of nitric oxide, this raises the possibility that there is a component of the vasorelaxation to acetylcholine which cannot be accounted for by the generation of nitric oxide. It is known that EDRF, like the nitrovasodilators, stimulates the soluble guanylyl cyclase in smooth muscle cells (Rapoport *et al.*, 1983; Förstermann *et al.*, 1986c; Martin & White, 1987), but if EDRF is identical to nitric oxide it would be expected that the same maximum responses would be obtained for nitrovasodilators and EDRF producing vasodilators. Since this is not the case in the superior mesenteric arterial bed, the greater effectiveness of the endothelium-dependent relaxation might be explained by the maximal rate of release of nitric oxide being less than the maximum rate of generation of EDRF/nitric oxide by the endothelium in response to acetylcholine. Alternatively, there might be a mechanism in addition to the stimulation of guanylyl cyclase or the enzyme is more responsive to the factor or factors produced by the endothelium than to nitric oxide.

We also wished to confirm that the pretreatment with phenobarbitone induced cytochrome P450 in the mesenteric preparation. This cytochrome is widely distributed and was described in mammalian vascular tissue by Juchau *et al.* (1976). This vascular cytochrome P450 has been localized to the endothelium (Abraham *et al.*, 1985; Pinto *et al.*, 1986). The results of this study show that the cytochrome P450 in the mesentery preparation is phenobarbitone-inducible. Also, the increase in content of the cytochrome was very similar in size to the 4 fold increase in rat aortic microsomes found by Finnen *et al.* (1986) after phenobarbitone pretreatment. The increase in the mesenteric cytochrome P450 content was almost entirely due to the 180% increase in microsomal protein content, rather than to an increase in the proportion of the protein that consists of the cytochrome.

The products of cytochrome P450-mediated metabolism of arachidonic acid are known to include monohydroxy and keto derivatives (Capdevila *et al.*, 1981) and four novel epoxides (Chacos *et al.*, 1982). Of these, the 5,6-epoxide has been shown to relax vascular smooth muscle (Schwartzman *et al.*, 1985; Finnen *et al.*, 1986; Carroll *et al.*, 1987; Proctor *et al.*, 1987). Prostaglandins do not appear to be produced (Capdevila *et al.*, 1981), but one group has suggested that cytochrome P450 is functional as prostacyclin synthetase (Ullrich *et al.*, 1981; Graf & Ullrich, 1982). In order to eliminate the possibility that the shift in the acetylcholine log dose-response curve after phenobarbitone pretreatment was due to increased activity of cyclo-oxygenase products, we examined the action of indomethacin, which would inhibit the cyclo-oxygenase and thus limit the production of endoperoxides, in rats pretreated with the barbiturate and found it had no effect. Thus the increase in the potency of acetylcholine as an endothelium-dependent vasodilator is not due to it stimulating the production of vasodilator prostaglandins.

The present results show that pretreatment of rats with phenobarbitone increases the potency of acetylcholine as an endothelium-dependent vasodilator (as assessed by its ability to oppose pressor responses to noradrenaline) in the isolated superior mesenteric arterial bed of the rat. This increase in potency is not accompanied by changes in the sensitivity of the vascular smooth muscle to sodium nitroprusside, which suggests the effect is at the level of the endothelium. The pretreatment produces an increase in mesenteric cytochrome P450 and the increased potency of acetylcholine is not due to changes in the production of cyclo-oxygenase products.

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