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# Endothelium-independent relaxation of aortic rings by the nitric oxide synthase inhibitor diphenyleneiodonium

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**1** The flavoprotein binder diphenyleneiodonium (DPI) is a potent, irreversible inhibitor of nitric oxide synthase (NOS), but produces only a transient pressor response following systemic administration to animals, despite evidence of persistent NOS inhibition. To characterize further the effects of DPI on vascular tone, isometric tension was recorded from rat isolated aortic rings mounted between steel wires in an organ bath.

**2** The NOS inhibitor $N^G$-nitro-L-arginine methyl ester (L-NAME, 1 mM) initiated an additional contraction of prostaglandin $F_{2\alpha}$-preconstricted rings with endothelium which was sustained throughout the period of L-NAME exposure ($+234\pm39\%$ at 15 min). In contrast, addition of DPI (5 $\mu$M) to rings with endothelium produced a transient initial contraction ($+111\pm27\%$ at 2 min) followed by a more sustained relaxation ($-27\pm19\%$ at 15 min, $P<0.001$ vs L-NAME).

**3** The contraction to DPI was also observed in rings without endothelium, was abolished by L-NAME pretreatment, and was unaffected by the $\alpha$-adrenoreceptor inhibitor prazosin. Relaxation in response to DPI was not inhibited by endothelium removal or by pretreatment with either L-NAME or with the ATP-sensitive potassium channel blocker glibenclamide.

**4** The endothelium-independent relaxation to DPI was inhibited at 23°C and its time course was delayed by pretreatment with the guanylate cyclase inhibitor methylene blue.

**5** Thus, in addition to a transient initial contraction due to NOS inhibition, DPI produces an endothelium-independent, temperature-dependent relaxation which appears in part due to activation of guanylate cyclase. This relaxant effect of DPI may explain the transient nature of its pressor effect *in vivo* despite sustained NOS inhibition.

**Keywords:** Diphenyleneiodonium; vasodilatation; endothelium; nitric oxide synthase; guanylate cyclase

## Introduction

The vasodilator nitric oxide (NO) is produced from L-arginine through the action of the NO synthase (NOS) (Moncada & Higgs, 1993), an enzyme which requires NADPH, FAD, FMN, haem (ferroprotoporphyrin IX), tetrahydrobiopterin, and $O_2$ (Nathan, 1992; Schmidt *et al*., 1992). Studies of the role of NO and of the regulation of nitric oxide synthase (NOS) in biological systems have been facilitated by the use of NOS inhibitors. While the substituted arginine analogues like $N^G$-nitro-L-arginine methyl ester (L-NAME) have been most frequently studied, five classes of NOS inhibitors have so far been described (Nathan, 1992). Among these classes are the flavoprotein binders which are believed to inhibit NOS by binding to the flavine-containing reductase domain of the enzyme which transfers electrons from NADPH to NOS haem (Stuehr & Ikeda-Saito, 1992; Klatt *et al*., 1993).

Recently, the flavoprotein binder and NADPH oxidase inhibitor diphenyleneiodonium (DPI) and its analogues were found to be potent inhibitors of NOS in macrophages (Stuehr *et al*., 1991) and in rabbit and rat aorta (Stuehr *et al*., 1991; Wang *et al*., 1993). Irreversible, time- and temperature-dependent inhibition of semipurified macrophage NOS by DPI has been observed with an $IC_{50}$ of 0.05 $\mu$M (Stuehr *et al*., 1991), while inhibition of acetylcholine (ACh)-induced relaxation in rat (Wang *et al*., 1993) and rabbit (Stuehr *et al*., 1991) isolated aortic rings has been obtained with an $IC_{50}$s of 0.18 and 0.3 $\mu$M respectively. The $N^G$-substituted analogues of L-arginine also inhibit endothelium-dependent relaxation to ACh in the rat aorta, but appear to be less potent (Rand & Li, 1993). Further, the effects of the arginine analogues can be reversed by exogenous L-arginine (Wang & Pang, 1993), while those of DPI can be blocked by pretreatment with NADPH and FAD, but not by L-arginine (Wang *et al*., 1993).

In addition to their different chemical structures, mechanisms of action, and relative potencies, other differences between DPI and the arginine analogues have been observed. While both classes of NOS inhibitors block the hypotensive response to ACh when administered intravenously to intact animals (Rees *et al*., 1989; Wang *et al*., 1993), different haemodynamic effects are observed following systemic administration of each (Wang & Pang, 1993). In contrast to the sustained pressor response which follows systemic administration of the arginine analogues (Wang & Pang, 1990; Wang *et al*., 1993), the pressor response to DPI is transient (Wang *et al*., 1993; Wang & Pang, 1993). Indeed, while DPI inhibits endothelium-dependent relaxations of isolated aortic rings to ACh for at least 4 h and suppresses ACh-induced vasolidatation for at least 2 h after intravenous administration in rats (Wang *et al*., 1993), the pressor response is no longer evident after just 4 min (Wang *et al*., 1993; Wang & Pang, 1993). DPI also appears to have effects on responses to contractile agonists. Low concentrations ($<3$ $\mu$M) have been shown to either have no effect (Rand & Li, 1993) or slightly potentiate (Wang *et al*., 1993) contractions of the rat aorta to phenylephrine, whereas at higher concentrations (10 $\mu$M) the contractile re-

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sponse may be reduced (Rand & Li, 1993). In the rat anococcygeus muscle, DPI similarly does not affect or slightly potentiates guanethidine-induced tone at low concentrations ($<3$ $\mu$M) while decreasing tone at higher concentrations (10 $\mu$M) (Rand & Li, 1993). Thus, DPI is an example of a novel class of NOS inhibitors, but it also appears to have other effects on vascular tone which may be unrelated to NOS inhibition. We therefore studied the effects of DPI, in rat isolated aortic rings, which are not related to its inhibition of NOS.

## Methods

### *Vessel preparation*

Two-to-three month old male Sprague-Dawley rats were killed by the intraperitoneal administration of sodium pentobarbitone (30–50 mg) in accordance with institutional guidelines in a manner that was as humane as possible. The thoracic aortae were removed and were placed in 95% $O_2$/5% $CO_2$-gassed Krebs-Henseleit buffer (consisting of (in mM): NaCl 119.0, $NaHCO_3$ 25, D-glucose 10.0, KCl 5.0, $CaCl_2$ 2.0, $MgSO_4$ 1.2 and $NaHPO_4$ 1.2) and dissected free of periadventitial fat and connective tissue with care taken to avoid touching the luminal surface. Each aorta was cut into six rings, each approximately 3 mm in length. In some rings, the endothelium was denuded by gently rubbing with a blunt 18 gauge hypodermic needle. The rings were mounted between horizontal stainless steel wires in a 25 ml organ bath containing Krebs-Henseleit buffer gassed with 95% $O_2$/5% $CO_2$. The lower wire was stationary and the upper wire attached to a force-displacement transducer (Gould Electronics, Cleveland, Ohio) for measurement of isometric tension. The output from the force transducer was recorded on a Gould Model 2400S recorder. The aortic rings were stretched progressively to achieve a resting tension of 3.0 g, determined to be the optimal resting tone for the rat aorta under these experimental conditions (defined as the minimum tension facilitating the development and maintenance of maximum contraction to 30 mM KCl). Vessels were equilibrated for 1½–2 h in the organ baths before each experiment unless otherwise noted.

### *Experimental protocol*

After the resting tension of aortic rings with and without endothelium had been adjusted to 3.0 g, the contractile response to 30 mM KCl was determined. This was maintained for approximately 15 min, after which the vessels were again washed. After stabilization at resting tension, the vessels were preconstricted with prostaglandin (PG)$F_{2\alpha}$ to achieve a maximal contractile response based on concentration-response relationships performed previously under identical experimental conditions. After $PGF_{2\alpha}$ constriction, the integrity of the endothelium was determined in each ring by assessing the response to ACh (10 $\mu$M) before the experimental protocol was started. In some experiments, rings were exposed to ACh after pretreatment with 5 $\mu$M DPI (diphenyleneiodonium sulphate) or 1 mM L-NAME and $PGF_{2\alpha}$ preconstriction. DPI was employed at a concentration of 5 $\mu$M since previous studies showed that micromolar concentrations of DPI inhibit endothelium-dependent relaxation of rabbit (Stuehr *et al*., 1991) and rat (Rand & Li, 1993; Wang *et al*., 1993) aortic rings. In other studies, rings were preconstricted with $PGF_{2\alpha}$ to 50% of the maximal contractile response ($EC_{50}$) based on concentration-response relationships previously determined. The previously-determined $EC_{50}$ was used rather than an $EC_{50}$ determined from a concentration-response relationship in each ring on each experimental day because repeated exposure to $PGF_{2\alpha}$ alters the concentration-response relationship to subsequent agonist exposures in this vessel. After the response had reached a plateau at the $EC_{50}$, the change in tension was assessed following the addition of DPI or L-NAME to the bath. Experiments were also performed in which DPI was added to endothelium-denuded rings which were maximally constricted with either $PGF_{2\alpha}$ or 5-hydroxytryptamine (5-HT) and pretreated with either the soluble guanylate cyclase inhibitor, methylene blue, or the ATP-sensitive potassium channel blocker, glibenclamide.

### *Materials*

$PGF_{2\alpha}$, ACh, L-NAME, 5-HT, methylene blue, glibenclamide, dimethysulphoxide (DMSO) and cromakalim were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Glibenclamide was dissolved in Krebs-Henseleit buffer containing DMSO (final concentration 0.1 vol%) and employed at a concentration which inhibited relaxation to the ATP-sensitive potassium channel opener cromokalim (0.2 $\mu$M). DPI was obtained from Colour Your Enzyme (Ontario, Canada).

### *Data analysis*

Data are presented as the mean$\pm$s.e.mean. Averaged data were obtained at 30 s, 1 min and every minute thereafter for the remainder of exposure since a review of individual tension recordings showed that the response to DPI or L-NAME was rapid, with an onset within 60 s. On each experimental day, each group consisted of $\geqslant 3$ rings from each animal, and rings from at least two experimental days were studied for each group. Averaged data are expressed as the % change in the contraction to $PGF_{2\alpha}$. When the experimental results were compared to control within a single ring, a Student's $t$ test for paired analysis was used; when results in different rings were compared, a Student's $t$ test for unpaired variables was performed. Comparisons between concentration-response relationships were made by an analysis of variance (ANOVA). A repeated measures ANOVA was used to compare tension recordings from different experimental groups. A difference was considered significant when $P<0.05$.

## Results

### *Effect of NOS inhibition on endothelium-dependent vascular tone*

Pretreatment with either L-NAME or DPI blocked relaxation to ACh in $PGF_{2\alpha}$-preconstricted, endothelium-intact rings (Figure 1b and c) and this inhibition was sustained for at least 3 h in the presence of either DPI or L-NAME (data not shown). Inhibition of ACh-induced relaxation by DPI persisted for at least 2 h after washout. When added at resting tension, both L-NAME and DPI initiated a small constriction of rings with endothelium at 15 min of exposure ($3.03\pm0.02$ to $3.11\pm0.02$, $n=9$ for L-NAME; $3.04\pm0.05$ to $3.10\pm0.05$, $n=11$ for DPI, $P<0.01$ for each).

When either L-NAME or DPI was added to partially-preconstricted rings with endothelium (Figure 1d and e), a rapidly-developing additional contraction occurred, consistent with removal of tonic NO production. While the onset and magnitude of the early contraction initiated by L-NAME and DPI were similar ($+145\pm38$% for L-NAME vs $+92\pm24$% for DPI at 1 min, NS), the L-NAME contraction was sustained throughout the period of exposure ($+234\pm39$% at 15 min), whereas that due to DPI was transient and followed by a progressive relaxation to a minimum tension of $-27\pm19$% at 15 min of continued exposure ($n=11$, $P<0.001$ vs L-NAME). Thus, while both NOS inhibitors similarly prevented endothelium-dependent vasodilatation to ACh and induced vasoconstriction at resting tension, the addition of DPI to $PGF_{2\alpha}$-preconstricted rings revealed a vasodilatation not found with L-NAME that may account for the different duration of the constriction each inhibitor initiated.

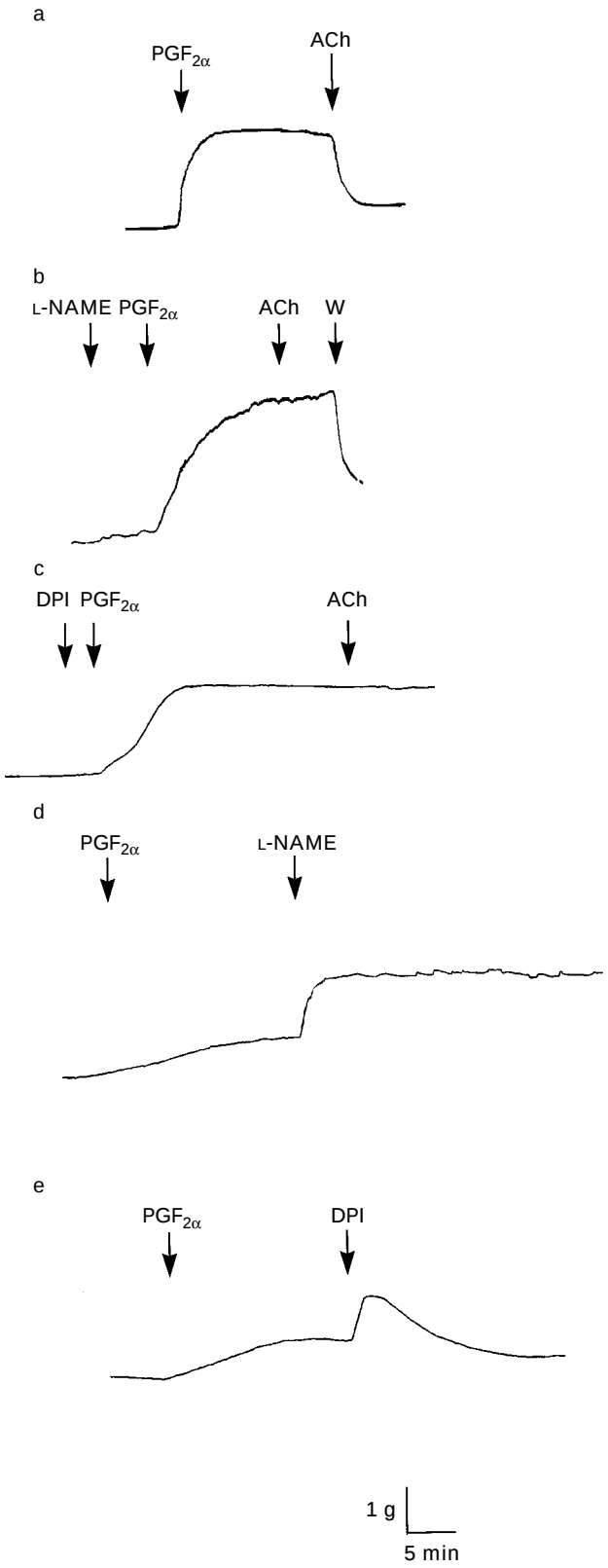


**Figure 1** Effect of the NOS inhibitors L-NAME and diphenyleneiodonium (DPI) on endothelium-dependent relaxation to ACh and on $PGF_{2\alpha}$-preconstricted aortic rings. (a) Representative recording of isometric tension from a rat isolated aortic ring with endothelium preconstricted with $PGF_{2\alpha}$ (3 $\mu$M) and then exposed to 10 $\mu$M ACh. (b) Representative recording of isometric tension from a rat isolated aortic ring with endothelium during exposure to 10 $\mu$M ACh following pretreatment with the NOS inhibitor L-NAME (1 mM) and preconstriction with $PGF_{2\alpha}$ (3 $\mu$M). Following exposure to ACh during the wash (W), all drugs were removed from the bath and fresh buffer entered the chamber. Similar results were obtained in a further 7 experiments. (c) Representative recording of isometric tension from a rat isolated aortic ring with endothelium during exposure to 10 $\mu$M ACh following pretreatment with 5 $\mu$M DPI and preconstriction with $PGF_{2\alpha}$ (3 $\mu$M). DPI inhibited relaxation to ACh under these conditions. Similar results were obtained in a further 12 experiments. (d) Representative recording of isometric tension from a rat isolated aortic ring with endothelium partically preconstricted to 50% of the maximal contractile response to $PGF_{2\alpha}$ (1 $\mu$M) and then exposed to 1 mM L-NAME. Similar results were obtained in a further 10 experiments. (e) Representative recording of isometric tension from a rat isolated aortic ring with endothelium exposed to 5 $\mu$M DPI following half-maximal preconstriction to $PGF_{2\alpha}$ (1 $\mu$M). Similar results were obtained in a further 12 experiments.

### *Effect of increasing concentrations of DPI on preconstricted aortic rings*

Figure 2 shows the effect of cumulative additions of DPI to the organ bath containing an endothelium-intact aortic ring partially-preconstricted to 50% of the maximal contractile response to $PGF_{2\alpha}$. Five concentrations of DPI (5 nM to 10 $\mu$M) were examined. The threshold concentration for vasoconstriction was 50 nM. Both a further, transient contraction and a more sustained relaxation were noted at 0.5 $\mu$M. No additional constriction was observed at higher concentrations.

### *Effects of α-adrenoreceptor blockade on the DPI contraction of aortic rings*

Since the transient pressor response to DPI in intact animals is attenuated by α-adrenoceptor antagonists (Wang & Pang, 1993), we examined whether pretreatment with the α-adrenoceptor antagonist prazosin affected the initial, transient contraction to DPI in isolated rings. While this concentration of prazosin abolished the contraction to phenylephrine (Figure 3b), it had no effect on the onset or magnitude of the transient contraction to DPI in $PGF_{2\alpha}$-preconstricted rings ($+41\pm5\%$ at 1 min, $n=8$, NS vs no prazosin).

### *Endothelium-dependent and -independent effects of DPI in aortic rings*

DPI induced an initial, transient contraction of $PGF_{2\alpha}$-preconstricted rings with and without endothelium (Figure 4) with a maximal effect at 2 min ($+111\pm27\%$ for rings with endothelium and $+55\pm17\%$ for rings without endothelium). This contraction was abolished by L-NAME pretreatment. The initial, transient contraction was followed by a more prolonged relaxation with a similar time course in both rings with and without endothelium. While L-NAME pretreatment of rings with endothelium abolished the initial contraction to DPI, it did not affect the subsequent relaxation ($-67\pm15\%$ for L-NAME-pretreated rings with endothelium vs $-71\pm9\%$ for endothelium-denuded rings not pretreated with L-NAME, both at 15 min, NS).

### *Temperature-dependence of the effect of DPI in aortic rings*

In order to examine the effect of temperature on the response to DPI in rat aorta, $PGF_{2\alpha}$-preconstricted rings were exposed to DPI at either 37°C or 23°C. As shown in Figure 5a, DPI initiated a rapid further contraction of preconstricted rings at both temperatures. While this contraction was sustained throughout the period of DPI exposure at 23°C ($+59\pm22\%$ at 15 min), it was followed by a relaxation at 37°C ($-27\pm19\%$ at 15 min, $P<0.01$ vs 23°C). This suggests that the effect of DPI on the endothelium is greater in magnitude or duration at 23°C and obscures an endothelium-independent effect or that the relaxant effect of DPI is not present at the lower temperature. To differentiate between these possibilities, DPI was applied at both temperatures to $PGF_{2\alpha}$-preconstricted rings without endothelium (Figure 5b) and to endothelium-intact

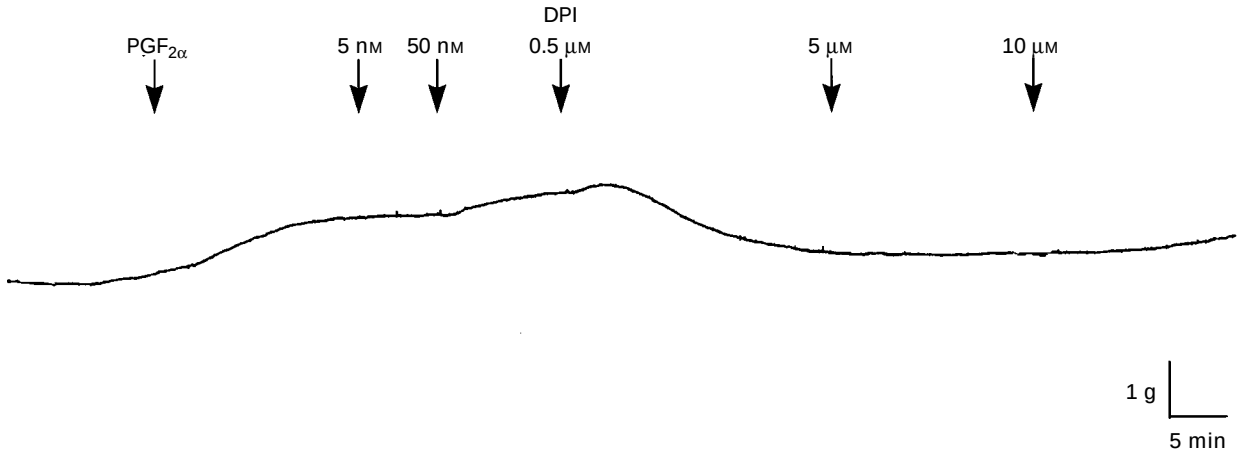


**Figure 2** Representative recording of isometric tension from a rat isolated aortic ring with endothelium exposed to increasing concentrations of diphenyleneiodonium (DPI) after half-maximal preconstriction with $PGF_{2\alpha}$ (1 $\mu$M). Cumulative concentrations of DPI are noted above the bold arrows. Similar results were obtained in a further 6 experiments.

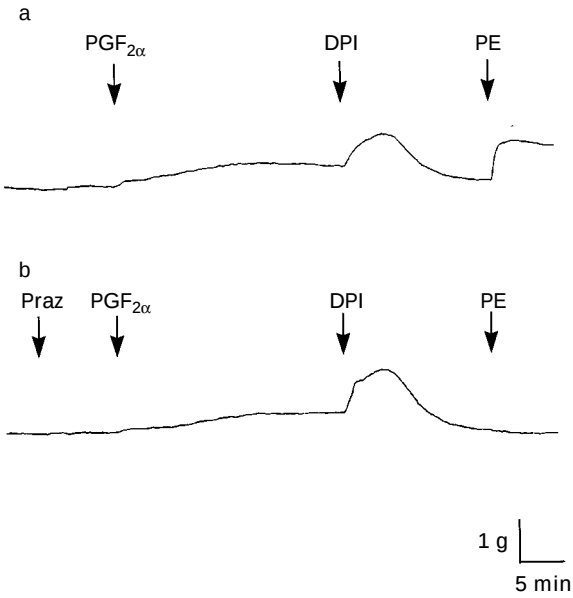


**Figure 3** Representative recording of isometric tension from a rat aortic ring with endothelium half-maximally constricted with $PGF_{2\alpha}$ (1 $\mu$M) and then exposed sequentially to 5 $\mu$M diphenyleneiodonium (DPI) and 5 $\mu$M phenylephrine (PE) in the absence (a), and presence (b) of the $\alpha$-adrenoceptor blocker prazosin (Praz, 3 $\mu$M). Similar results were obtained in a further 7 experiments.

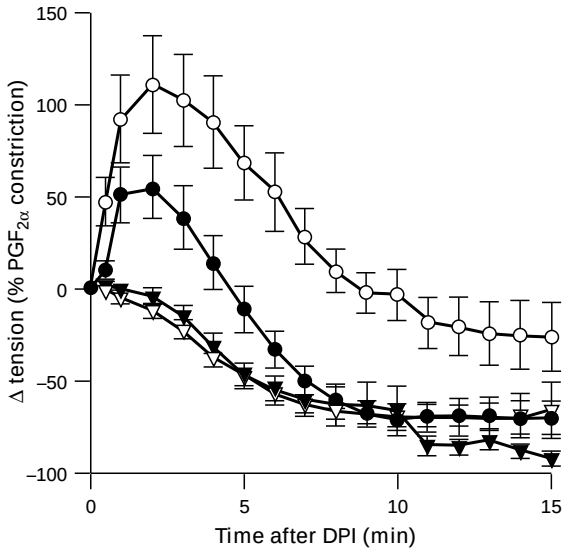


**Figure 4** Endothelium-dependent and -independent effects of diphenyleneiodonium (DPI). Averaged data of isometric tension recordings from rat aortic rings half-maximally preconstricted with $PGF_{2\alpha}$ (1 $\mu$M) and then exposed to 5 $\mu$M DPI. DPI was added to the organ bath at time zero and was present throughout the 15 min. When present, 1 mM L-NAME ($\triangledown$, $\blacktriangledown$) was added at least 15 min before the addition of DPI to the organ bath. DPI produced an initial transient contraction and a subsequent sustained relaxation in both endothelium-intact ($\bigcirc$) and endothelium-denuded ($\bullet$) aortic rings. L-NAME pretreatment inhibited the initial contraction in both endothelium-intact ($\triangledown$) ($P<0.001$) and endothelium-denuded ($\blacktriangledown$) ($P<0.05$) rings. The change in tension is expressed as the % change in the contraction to $PGF_{2\alpha}$ and is presented as mean$\pm$s.e.mean (vertical lines) as described in the text. Averaged data from rings with endothelium ($\bigcirc$, $n=13$), without endothelium ($\bullet$, $n=29$), and from L-NAME-pretreated rings with ($\triangledown$, $n=9$) and without ($\blacktriangledown$, $n=20$) endothelium are shown.

rings pretreated with L-NAME (Figure 5c). As shown in (b), the initial, transient contraction of rings without endothelium was slightly blunted at 23°C compared with 37°C, although this difference was not significant. However, a temperature-dependent effect on the endothelium-independent relaxation to DPI was observed, as this relaxation was inhibited at 23°C ($P<0.05$ vs 37°C). At 37°C, endothelium-denuded rings were fully relaxed by 10 min of continued DPI exposure ($-72\pm6\%$, $n=29$), whereas tension was $-15\pm3\%$ at this time at 23°C ($n=6$, $P<0.001$ vs 37°C). The effect of temperature on the relaxation of aortic rings was most apparent in L-NAME-pretreated rings with endothelium (Figure 5c). In these experiments, the initial contraction to DPI was abolished by pretreatment with the NOS inhibitor L-NAME at both temperatures. As in the endothelium-denuded rings in Figure 5b, full relaxation to DPI was already seen at 10 min of continued exposure at 37°C ($-70\pm6\%$, $n=9$), but did not occur at 23°C ($n=7$, $P<0.001$). Thus, the contraction in response to DPI appeared to be due to inhibition of NOS, since it was abolished by L-NAME pretreatment at both temperatures. No significant effect of temperature on this initial contraction was observed in these studies. In contrast, a significant temperature-dependent effect on relaxation to DPI was observed, with inhibition of relaxation noted at 23°C.

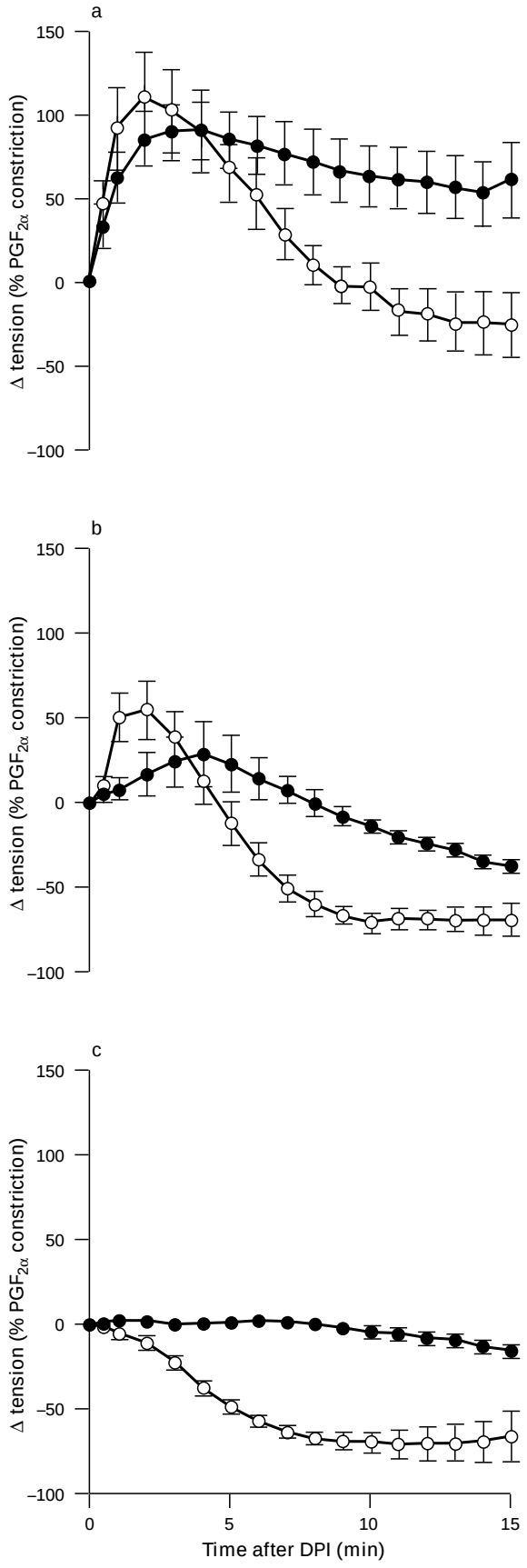


**Figure 5** Temperature-dependence of the effect of diphenyleneiodonium (DPI). (a) Averaged data of isometric tension recordings from rat aortic rings with endothelium half-maximally preconstricted with $PGF_{2\alpha}$ (1 $\mu$M) and then exposed to 5 $\mu$M DPI at either 37°C (○) or 23°C (●). DPI was added to the organ bath at time zero and was present throughout the 15 min. The change in tension is expressed as % change in the contraction to $PGF_{2\alpha}$ and is presented as the mean ± s.e.mean (vertical lines) of 13 rings. (b) Averaged data of isometric tension recordings from rat aortic rings without endothelium half-maximally preconstricted with $PGF_{2\alpha}$ (1 $\mu$M) and then exposed to 5 $\mu$M DPI at either 37°C (○) or 23°C (●). DPI was added to the organ bath at time zero and was present throughout the 15 min. The change in tension is expressed as % change in the contraction to $PGF_{2\alpha}$ and is presented as the mean ± s.e.mean of 29 rings at 37°C and of 6 rings at 23°C. (c) Averaged data of isometric tension recordings from rat aortic rings with endothelium pretreated with 1 mM L-NAME half-maximally preconstricted with 1 $\mu$M $PGF_{2\alpha}$ and then exposed to 5 $\mu$M DPI at either 37°C (○) or 23°C (●). DPI was added to the organ bath at time zero and was present throughout the 15 min. The change in tension is expressed as % change in the contraction to $PGF_{2\alpha}$ and is presented as the mean ± s.e.mean of 7–9 rings.

### *Effect of glibenclamide on the relaxation to DPI in rings without endothelium*

To determine whether DPI relaxes aortic rings by affecting the ATP-sensitive potassium channel, we examined the effect of glibenclamide on the response to DPI in endothelium-denuded, maximally preconstricted rings. Rings were preconstricted with 5-hydroxytryptamine (5-HT, 20 $\mu$M) instead of $PGF_{2\alpha}$ since glibenclamide inhibits contractions to $PGF_{2\alpha}$ but does not affect contractions to 5-HT (Zhang & Cook, 1994). Since glibenclamide was dissolved in DMSO, the relaxation in response to DPI in glibenclamide-pretreated rings was compared to rings in which an identical concentration of DMSO (0.1 vol%) was in the bath. The concentration of glibenclamide (10 $\mu$M) used for these studies abolished the relaxation to the ATP-sensitive potassium channel opener cromokalim (0.2 $\mu$M, data not shown). Following pretreatment with glibenclamide (Figure 6, $n=11$), relaxation to DPI was unchanged compared to control rings ($n=8$, NS) exposed only to vehicle (DMSO).

### *Effect of methylene blue on the relaxation of endothelium-denuded rings to DPI*

To determine whether the relaxation to DPI is mediated by activation of guanylate cyclase, rings without endothelium were pretreated with the soluble guanylate cyclase inhibitor methylene blue (MB, 10 $\mu$M) prior to the preconstriction to $PGF_{2\alpha}$. Following MB-treatment, the time course of relaxation to DPI was prolonged (Figure 7). While rings were fully relaxed ($-65\pm6$%, $n=6$) 10 min after DPI addition in control rings, only ~50% relaxation ($-30\pm5$%, $P<0.05$ vs control) was noted at this time in rings pretreated with MB. In other experiments, $PGF_{2\alpha}$-preconstricted rings without endothelium were relaxed with 5 $\mu$M DPI. DPI was then washed for either 15 min or 2 h and then MB was added to determine whether the apparent effects of DPI on guanylate cyclase are reversible. Application of MB contracted the DPI-relaxed rings by $46\pm19$% of the initial relaxation to DPI ($n=4$) after a 15 min wash and $27\pm17$% following a 2 h wash ($n=3$).

## Discussion

This study shows that the flavoprotein binder DPI, recently found to be one of a new class of NOS inhibitors (Stuehr *et al*., 1991; Wang *et al*., 1993), also produces endothelium-independent relaxation of isolated aortic rings which is unrelated to its effect on NOS. These results confirm those of others (Stuehr *et al*., 1991; Rand & Li, 1993; Wang *et al*., 1993) that DPI, like the NOS inhibitor L-NAME, inhibited endothelium-dependent vasodilatation to ACh. Both L-NAME and DPI initiated a small constriction of rings with endothelium when added at resting tension, implying that basal NO release (Tsukahara *et al*., 1993) is inhibited to a similar extent by both NOS inhibitors. However, when applied to preconstricted aortic rings, these NOS inhibitors have distinct effects. While

L-NAME produces a further sustained contraction of partially preconstricted rings, the contraction in response to DPI was transient and was followed by a prolonged relaxation. The threshold concentration for the relaxation to DPI was higher than that for the initial contraction. The contraction initiated by DPI appears to be due to removal of NO, since it was abolished by L-NAME pretreatment. In contrast, the endothelium-independent relaxation to DPI is unrelated to NO, since it was not affected by L-NAME pretreatment. This relaxant effect is at least partially mediated by activation of guanylate cyclase, since its time course was delayed by pretreatment with methylene blue. While relaxation to DPI was inhibited at 23°C, when metabolic demand is likely to be lower than at 37°C, it does not appear to be mediated by activation of the ATP-sensitive potassium channel, as it was unaffected by pretreatment with glibenclamide.

The transient contraction of $PGF_{2\alpha}$-preconstricted rings in response to DPI, in contrast to the sustained contraction to L-NAME, is reminiscent of the respective effects of these NOS inhibitors on blood pressure following systemic administration. While the substituted arginine analogues produce sustained pressor responses in whole animals (Aisaka *et al*., 1989; Rees *et al*., 1989; Wang & Pang, 1990), intravenous bolus injections of DPI in rats produce increases in mean arterial pressure (MAP) which last only about 4 min (Wang *et al*., 1993; Wang & Pang, 1993), despite persistent (>2 h) inhibition of ACh-induced vasodilatation (Stuehr *et al*., 1991; Wang *et al*., 1993). In the present study, DPI initiated a contraction of $PGF_{2\alpha}$-preconstricted rings which resolved after approximately 8 min (see Figure 4), even though inhibition of ACh-induced relaxation persisted for hours after DPI administration (data not shown).

The reason why DPI produced sustained inhibition of endothelium-dependent vasodilatation but only a transient pressor response is not understood, but it has been suggested that the effect of DPI on blood pressure may not be due to inhibition of NOS alone (Wang *et al*., 1993). For example, Wang & Pang (1993) attributed the pressor effect of DPI to indirect activation of the sympathetic nervous system. In the present study, the initial contraction of isolated aortic rings in response to DPI is not mediated through $\alpha_1$-adrenoceptor stimulation, since it was not inhibited by prazosin.

Both endothelium-intact and endothelium-denuded rings demonstrated an initial, transient contraction to DPI following $PGF_{2\alpha}$-preconstriction. This effect appears to be mediated through inhibition of NO synthesis or release in both endothelial and vascular smooth muscle cells, since it was abolished by pretreatment with L-NAME. The initial contraction of rings both with and without endothelium was also observed when L-NAME was added to $PGF_{2\alpha}$-preconstricted rings, and this effect was abolished by DPI pretreatment. Charpie & Webb (1993) previously documented contraction of the endothelium-denuded rat aorta to the NOS inhibitor $N^{\omega}$-nitro-L-arginine (L-NNA) and felt this was consistent with withdrawal of smooth muscle NO synthesis or release. These authors also showed that the contractile response to ACh in endothelium-denuded aortic segments was greater when L-NNA was present, suggesting that vascular smooth muscle-derived NO limited the contractile activity to ACh. Schini and Vanhoutte (1991) also found evidence of NOS activity in vascular smooth muscle, since L-arginine evoked concentration- and time-dependent relaxations of endothelium-denuded rat aortic rings after 2 h of incubation in physiological salt solution and contractions of endothelium-denuded rings to phenylephrine were enhanced in the presence of either nitro-L-arginine or methylene blue.

Most evidence suggests that vascular smooth muscle NOS activity is inducible and not constitutive. High levels of inducible NOS (iNOS) mRNA have been detected in human cultured aortic smooth muscle cells stimulated with cytokines and lipopolysaccharide (MacNaul & Hutchinson, 1993), but no constitutive endothelial cell NOS (ec-NOS) was detected in these smooth muscle cells under any of the conditions examined. In addition, Hansson *et al*. (1994) demonstrated NOS mRNA expression by *in situ* hybridization in the endothelium of the uninjured rat carotid artery, but not in medial smooth muscle cells. When experiments were performed to isolate, denude, mount, and preconstrict rat aortic rings within one

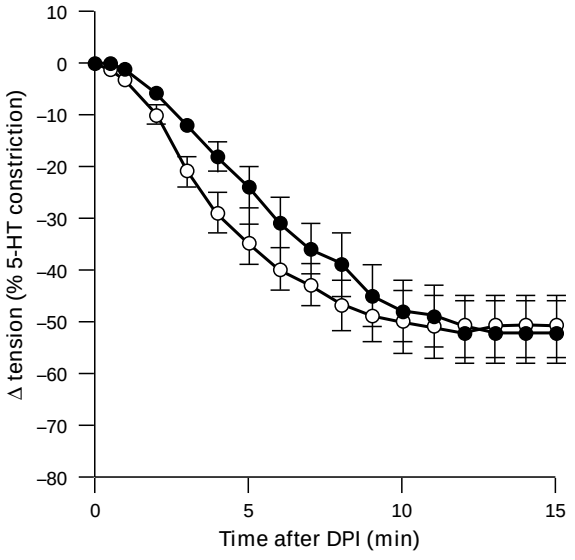


**Figure 6** Effect of glibenclamide pretreatment on the relaxant effect of diphenyleneiodonium (DPI). Averaged data of isometric tension recordings from endothelium-denuded rat aortic rings with (●) or without (○) pretreatment with the ATP-sensitive potassium channel blocker, glibenclamide (10 $\mu$M). Rings were maximally preconstricted with 5-hydroxytryptamine (5-HT, 20 $\mu$M) and then exposed to 5 $\mu$M DPI. DPI was added to the organ bath at time zero and was present throughout the 15 min. The change in tension is expressed as % change in the contraction to 5-HT and is presented as the mean ± s.e.mean (vertical lines) of 8–11 rings.

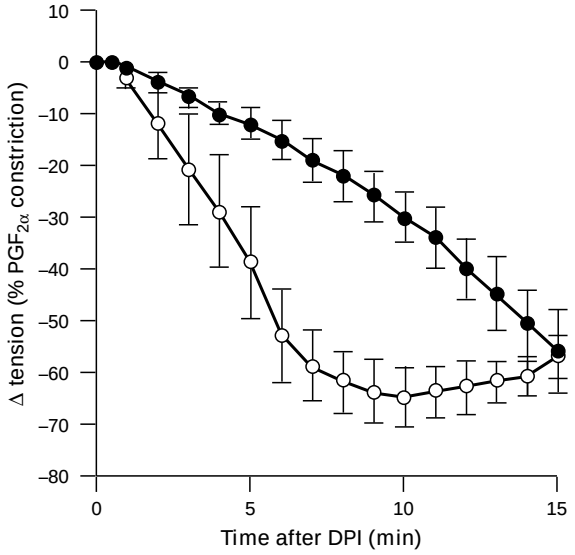


**Figure 7** Averaged data of isometric tension recordings from rat aortic rings maximally preconstricted with 4 $\mu$M $PGF_{2\alpha}$ and then exposed to 5 $\mu$M diphenyleneiodonium (DPI) either with (●, $n=10$) or without (○, $n=6$) pretreatment with 10 $\mu$M methylene blue, an inhibitor of soluble guanylate cyclase. DPI was added to the organ bath at time zero and was present throughout the 15 min. The change in tension is expressed as % change in the contraction to $PGF_{2\alpha}$ and is presented as mean ± s.e.mean (vertical lines) of 6 rings with and 10 rings without methylene blue pretreatment.

hour (without maintaining the rings at resting tension for 1½–2 h before the experiment was started), the additional constriction of endothelium-denuded rings to either DPI or L-NAME did not occur ($n=4$ for each, data not shown). The present work therefore suggests that, under the conditions employed for most of these experiments, the transient contraction of endothelium-denuded rings to DPI is due to inhibition of iNOS in rat aortic vascular smooth muscle as has been found previously (Beasley & McGuiggin, 1994).

The present studies with methylene blue suggest that a component of the relaxant effect of DPI is due to guanylate cyclase activation in vascular smooth muscle and that this persists for up to 2 h after washout of DPI. This is consistent with the findings of Pettibone *et al*. (1985) who showed that the DPI analogue, diphenyliodonium, stimulated guanylate cyclase activity in rat lung. These authors also found that diphenyliodonium decreased blood pressure and total peripheral resistance in anaesthetized dogs and suggested that this was due to stimulation of guanylate cyclase in vascular smooth muscle, since 1–10 $\mu$M diphenyliodonium relaxed rabbit isolated aorta with or without endothelium. The effects of DPI on guanylate cyclase activity and on guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels in intact arteries are complex. DPI may act directly to increase vascular smooth muscle cyclic GMP levels, while simultaneously decreasing cyclic GMP levels in vessels with intact endothelium (McGuire *et al*., 1994) through its inhibition of NOS. Although methylene blue is often used as a 'selective' inhibitor of soluble guanylate cyclase, it is important to note that MB has other effects, including the ability to generate superoxide anion (McCord & Fridovich, 1970) and to inhibit NOS (Mayer *et al*., 1993). It is unlikely that the effects of MB on DPI-induced relaxation are related to NOS inhibition, since DPI itself inhibits NOS. Pretreatment of rings with superoxide dismutase (SOD, 150 u ml$^{-1}$) did not alter the effect of MB on DPI-induced relaxation of $PGF_{2\alpha}$-preconstricted rings ($n=4$, data not shown), suggesting that generation of superoxide anion by MB is not involved in its effects on the relaxation induced by DPI.

The effect of DPI on mitochondrial respiration (Gatley & Sherrat, 1976) and the temperature-dependent inhibition of the relaxation to DPI shown in this study suggested the possibility that DPI has effects related to impaired ATP production in vascular smooth muscle. Like rotenone, DPI inhibits NADH oxidation by an effect on NADH-ubiquinone-1 oxidoreductase (Ragan & Bloxham, 1977) and previous studies have shown that rotenone and other inhibitors of oxidative energy production decrease the contractile response of the rat aorta to phenylephrine (Rodman *et al*., 1991). On the other hand, it has been pointed out (McGuire *et al*., 1994) that inhibition of oxidative metabolism would be expected to have a greater effect on force development to a contractile agonist rather than on the tonic phase of agonist-induced contraction, since ATP consumption is higher during force development than during force maintenance due to rapid cross-bridge cycling (Somlyo & Somlyo, 1992). Indeed, fully relaxed aortic rings in the presence of DPI still constricted to phenylephrine in the present study (Figure 3), and other authors have found that DPI does not inhibit force development to phenylephrine (Rand & Li, 1993; Wang *et al*., 1993) or noradrenaline (Stuehr *et al*., 1991). A fall in intracellular ATP concentration might result in activation of vascular smooth muscle ATP-sensitive potassium channels (Standen *et al*., 1989), which would result in a reduction in intracellular $Ca^{2+}$ concentration and vasorelaxation. Pretreatment with the ATP-sensitive potassium channel blocker glibenclamide did not inhibit the subsequent relaxation to DPI (Figure 6), indicating that the ATP-sensitive potassium channel is unlikely to be involved in the relaxant effect of DPI.

Thus, the present study shows that in addition to NOS inhibition, DPI stimulates endothelium-independent relaxation of aortic rings by a mechanism which may involve guanylate cyclase activation in vascular smooth muscle. Other actions of DPI, which could explain its relaxant effect, include inhibition of both $K^+$ and $Ca^{2+}$ currents in vascular smooth muscle (Weir *et al*., 1994) or intracellular acidification of vascular smooth muscle (Dodd-o *et al*., 1995) which may alter myofilament responsiveness to calcium. By further characterizing the *in vitro* effects of DPI, the present study may provide an insight into some of the biological actions of this NOS inhibitor *in vivo*.

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