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The *in vitro* pulmonary vascular effects of FK409 (nitric oxide donor): a study in normotensive and pulmonary hypertensive rats

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1 Vasorelaxant responses to the nitric oxide (NO) donor, FK409 ((±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide), were evaluated on precontracted isolated ring preparations of main pulmonary artery and intralobar pulmonary artery from rats.

2 On main pulmonary artery FK409 fully reversed the precontractions. Responses were inhibited by methylene blue but were independent of the endothelium. The potency ($-\log EC_{50}$) of FK409 was the same on preparations contracted with noradrenaline (7.62) or the thromboxane-mimetic, U44619 (7.63).

3 On intralobar pulmonary artery FK409 caused only 80% reversal of the precontraction and was 2 fold less potent than on main pulmonary artery. These differences in maximum response and potency between main and intralobar arteries are in keeping with previous findings with other NO donors.

4 Pulmonary hypertension was induced in rats by chronic exposure to hypoxia (10% O_2) for 1 or 4 weeks. Main pulmonary arteries from 1 week hypoxic rats had inherent tone and showed spontaneous contractile activity. In these arteries FK409 reversed not only the precontraction induced by noradrenaline but also the inherent tone. However, FK409 was 17 fold less potent than in control arteries, reflecting previous findings with other NO donors. Main pulmonary arteries from 4 week hypoxic rats had minimal inherent tone and were quiescent and FK409 was 4.5 fold less potent than in control arteries. In intralobar pulmonary arteries from 4 week hypoxic rats FK409 was 12 fold less potent than in controls.

5 Treatment of arteries with either (a) *in vitro* hypoxic conditions (P_{O_2} of solution in organ bath <10 mmHg) or (b) superoxide dismutase (SOD; 150 u ml^{-1}) together with catalase (1200 u ml^{-1}) significantly increased the potency of FK409 in preparations from hypoxic rats but had no effect on the potency in control preparations. Neither SOD nor catalase, alone, nor the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester, had any effect on the potency of FK409 in preparations from control or hypoxic rats.

6 It is concluded that the reduction in potency of FK409 seen in pulmonary arteries from rats with chronic hypoxic pulmonary hypertension may be due in part to the presence of one or more reactive oxygen species (either hydroxyl or superoxide plus hydrogen peroxide).

Keywords: Nitric oxide donor; FK409; main pulmonary artery; intralobar pulmonary artery; pulmonary hypertension; hypoxia; reactive oxygen species

Introduction

Nitric oxide (NO) donors have a potential therapeutic role in those cardiovascular diseases where the functional properties of the vascular endothelium are impaired (Lefer, 1993). Pulmonary hypertension is one disease in which there is often a dysfunctional endothelium accompanied by impaired release of endothelial NO (Dinh-Xuan *et al*., 1991; Celermajer *et al*., 1993; Dinh-Xuan, 1993). NO gas, administered by inhalation, has been used to lower selectively pulmonary artery pressure and pulmonary vascular resistance in a variety of pulmonary hypertensive patients (Pepke-Zaba *et al*., 1991; Roberts *et al*., 1992; Kinsella *et al*., 1992; Williamson *et al*., 1996). However, there are obvious limitations to the long-term use of inhaled NO gas, and NO donor drugs may be a practical alternative, especially if they can be given by inhalation to achieve pulmonary selectivity.

FK409, (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide, is a novel NO donor that was first isolated from the fermentation broth of *Streptomyces griseosporeus* (Hino *et al*., 1989). It releases NO from its structure spontaneously without requiring enzymic activation (Kita *et al*., 1994). The vasodilator properties of this drug have been described *in vitro* in a number of systemic arteries (Ohtsuka *et al*., 1990; Shibata *et al*., 1991; Yamada *et al*., 1991; Isono *et al*., 1993). Its potency has been found to vary in different systemic artery types and it is particularly potent in coronary vessels (Ohtsuka *et al*., 1990). Its actions on pulmonary blood vessels have not been described. Therefore the first aim of this study was to examine the vasorelaxant properties of FK409 on isolated preparations of main pulmonary artery and intralobar pulmonary artery from rats.

If any drug is to be of value in pulmonary hypertension it is important that it remains an effective vasorelaxant in pulmonary vessels that have undergone the changes associated with the development of this particular disease. Therefore the second aim of this study was to evaluate responses to FK409 in pulmonary artery preparations from rats with experimental pulmonary hypertension. In previous studies, another NO donor, nitroprusside, has been shown to relax pulmonary arteries taken from pulmonary hypertensive rats fully but to have reduced potency when compared with data obtained in control rats (Wanstall *et al*., 1992; Rodman, 1992; Crawley *et al*., 1992; Maruyama & Maruyama, 1994). Therefore experiments were carried out to determine whether a reduction in potency was likewise seen for FK409 in arteries from pulmonary hypertensive rats. A further aim of this study was to investigate whether any reduction in potency might be due to the presence in pulmonary hypertensive arteries of one or more reactive oxygen species which could reduce the half-life of the NO released from the drug.

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A preliminary account of some of these data was presented to a meeting of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists, Adelaide, December 1995 (Kaye & Wanstall, 1995).

Methods

Rats

Male Wistar rats, aged 8–9 weeks on the day of the experiment, were used. Some of the rats were housed in hypoxic chambers (10% oxygen) for 1 week or 4 weeks before the experiment to induce pulmonary hypertension (Wanstall *et al*., 1992). Control rats were housed in room air (21% oxygen).

On the day of the experiment each rat was anaesthestized with pentobarbitone (90 mg kg^{-1}, i.p.), the thorax was opened and heparin (2500 iu kg^{-1}) was injected into the right ventricle. A blood sample was taken for measurement of haematocrit. After removal of the main pulmonary artery and, in some experiments, the lung, the heart was removed. The right ventricle (RV) and left ventricle plus septum (LV + S) were weighed separately to allow calculation of the ratios RV/(LV + S) and RV/body weight. Increases in these two ratios in rats exposed to hypoxia were taken as evidence of right ventricular hypertrophy. Right ventricular hypertrophy is indicative of an elevation in pulmonary artery pressure (Ghodsi & Will, 1981; Wanstall & O'Donnell, 1992; Wanstall *et al*., 1992) and was therefore taken as evidence that rats exposed to hypoxia were pulmonary hypertensive.

Main pulmonary artery preparations

Single ring preparations (3 mm in length) of main pulmonary artery were set up in physiological salt solution (PSS) at 37°C, around two stainless steel wires in a vertical organ bath. The compositions of the PSS was (mM): NaCl 118, KCl 5.9, $CaCl_2$ 1.5, $MgSO_4$ 0.72, $NaHCO_3$ 25, glucose 11.7 and ascorbic acid 1.14 (95% O_2/5% CO_2; pH 7.4). In one series of experiments the endothelium was deliberately removed by gently rubbing the luminal surface of the preparations with small forceps. In all other experiments care was taken not to damage the endothelium. Force in the circular muscle was recorded isometrically with a Statham Universal Transducer (UC3 + UL5) attached to a micrometer (Mitutoyo, Tokyo, Japan). The resting forces for the preparations were 10 mN (control rats) or 20 mN (pulmonary hypertensive rats). These resting forces were selected, as in previous studies (Wanstall & O'Donnell, 1992; Wanstall *et al*., 1995a), to reflect the different *in vivo* pulmonary artery pressures in control (normotensive) and pulmonary hypertensive rats.

At the completion of the experiment, the distance between the two horizontal wires (with the preparation under the selected resting force, see above) was measured with the micrometer attached to the force transducer. The wet weight of the preparation (after blotting for 30 s between filter papers) was determined. The cross-sectional area of the preparation (mm^2), in the plane perpendicular to the direction of the applied force, was then calculated as described by Wanstall & O'Donnell (1992) from the formula:

$$\text{Cross sectional area} = w(hd)^{-1}$$

where h = the distance between the wires plus the diameters of the two wires (mm), w = wet weight (mg) and d = density = 1.06 mg mm^{-3} (Murphy, 1980). Since all preparations were the same length (3 mm), an increase in cross-sectional area reflected an increase in vessel wall thickness and hence the presence of vascular hypertrophy in the main pulmonary artery.

Intralobar pulmonary artery preparations

Intralobar pulmonary arteries (i.d. 500–650 μm) were dissected out from the left lung. Ring preparations (length 1.60–1.95 mm) with the endothelium intact were mounted on 40 μm diameter stainless steel wires in a small vessel myograph (Mulvany-Halpern type; Model 400A; JP Trading, Aarhus, Denmark). The tissue chamber of the myograph contained PSS at 37°C bubbled with 95% O_2/5% CO_2. The preparations were individually normalised to resting forces that corresponded to transmural pressures of 15 mmHg (control rats) and 30 mmHg (pulmonary hypertensive rats); these resting forces were: control rats 2.0 ± 0.06 mN ($n = 4$); hypoxic rats 4.2 ± 0.17 mN ($n = 4$). Active force was recorded isometrically. Vessel wall thickness was not calculated for intralobar arteries because the preparations were too small to be accurately weighed.

Experimental protocols

Preparations were allowed to equilibrate for 1 h. They were then contracted submaximally with noradrenaline or U46619 and, when the contraction was stable, acetylcholine (ACh, 1 μM) was added. A relaxant response to ACh indicated the presence of a functional endothelium. The few preparations from which the endothelium was deliberately removed did not relax to ACh. After washing with PSS, a contraction to K^+-depolarizing PSS (in which 80 mM NaCl was replaced with 80 mM KCl) was obtained to stabilize the preparations. The preparations were washed and allowed to relax. They were then contracted submaximally with a spasmogen (noradrenaline or the thromboxane mimetic U46619) and a cumulative concentration-response (relaxation) curve to FK409 was determined. In most experiments on main pulmonary artery the spasmogen was noradrenaline, as in a previous study (Wanstall & O'Donnell, 1992). In intralobar pulmonary artery preparations contractions to noradrenaline were small and very variable; consequently, in intralobar arteries, and also in the paired preparations of main pulmonary artery taken from the same rat, the spasmogen was U46619. The concentrations of spasmogens were: U46619, 30 nM and 300 nM on main and intralobar pulmonary arteries, respectively; noradrenaline, 0.1 μM (main pulmonary artery only). These concentrations were in the range EC_{70}–EC_{90}, and gave contractions that were smaller than the contraction to K^+-depolarizing PSS. The use of different concentrations of U46619 on the two types of artery preparation reflected the finding that U46619 was less potent (i.e. had a higher EC_{50}) and gave a smaller maximum response on intralobar artery than on main pulmonary artery.

In some experiments the FK409 concentration-response curve was obtained under conditions of *in vitro* hypoxia or in the presence of one or more of the following drugs: methylene blue (10 μM), N^G-nitro-L-arginine methyl ester (L-NAME, 10 μM), superoxide dismutase (SOD, 150 u ml^{-1}), catalase (1200 u ml^{-1}). *In vitro* hypoxia (P_{O_2} of PSS < 10 mmHg) was induced by bubbling the PSS in the tissue bath with 95% N_2/5% CO_2 instead of 95% O_2/5% CO_2 for 1 h before and during the noradrenaline contraction and FK409 curve, as described previously (Wanstall, 1994). Methylene blue and L-NAME were added 20 and 10 min, respectively, before the noradrenaline contraction, whereas SOD and catalase were added immediately before the contraction. Each of these drugs remained in the bath during the FK409 concentration-response curve.

Analysis of data

Relaxant responses to FK409 were measured from the level of the steady state contraction induced by noradrenaline or U46619. The responses were expressed as a percentage of the maximum relaxation to FK409 and were plotted against concentration of FK409 on a logarithmic scale. EC_{50} values (where EC_{50} is the concentration producing 50% of the maximum relaxation to FK409) were interpolated from these plots. The potency of FK409 was expressed as the negative log EC_{50}. Maximum relaxations to FK409 and relaxant responses to

ACh were expressed as 'percentage reversal' of the noradrenaline- or U46619-induced contractions, as appropriate.

Drugs and solutions

Acetylcholine chloride (ACh, Sigma); catalase (Sigma); FK409 (kindly donated by Fujisawa Pharmaceutical Co, Ltd, Japan), methylene blue (Sigma); (−)-noradrenaline acid tartrate (Sigma); N^G-nitro-L-arginine methyl ester (L-NAME; Sigma); superoxide dismutase (SOD; Sigma); U46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$; Sigma).

Solutions of drugs were prepared as follows: ACh (10 mM), catalase (120,000 u ml^{-1}), FK409 (10 mM), L-NAME (10 mM), methylene blue (10 mM), SOD (10,000 u ml^{-1}) in deionised water, noradrenaline (100 mM) in 10 mM HCl; U46619 (10 mM) in absolute ethanol. Dilutions, when required, were made in PSS.

Statistical analyses

Mean values were calculated from data obtained in preparations from a number (n) of different animals and are quoted together with their s.e.mean. The statistical significance of differences between mean values of maximum relaxant response expressed as 'percentage reversal' was assessed by Mann Whitney U test (values not necessarily normally distributed). Statistical differences between all other mean values were analysed by an appropriate parametric test as follows. Where 2 values were compared, Student's t test (unpaired values) or paired t test (paired values) was applied. Where more than 2 values were compared, data were analysed by one way analysis of variance (ANOVA) followed by the appropriate *post-hoc* test, viz. Dunnett's test (when various test values were compared with control values) or Tukey-Kramer test, as indicated.

Results

Vasorelaxant effects of FK409 on main and intralobar pulmonary arteries from rats

On main pulmonary artery from control rats, FK409 completely reversed the contractions induced by noradrenaline (Figure 1) or U46619 (Figure 2), i.e. maximum relaxation corresponded to 100% reversal. The potency (negative log EC_{50}) of FK409 was the same irrespective of the spasmogen used (Table 1). Relaxant responses to FK409 were significantly inhibited by 10 μM methylene blue (Figure 1; Table 1) but were not affected by removal of the endothelium (Table 1).

On intralobar pulmonary artery, maximum relaxation to FK409 was only 80±2.0% reversal ($n=4$; Figure 2), i.e. less than on paired preparations of main pulmonary artery taken from the same rats ($P<0.05$). The potency of FK409 was about 2 fold less in intralobar than in main pulmonary artery, but this difference did not reach statistical significance (Table 1).

Induction of pulmonary hypertension in rats

Chronic exposure of rats to 10% oxygen for 1 or 4 weeks resulted in retarded growth, polycythaemia, right ventricular hypertrophy and vascular hypertrophy of main pulmonary artery (Table 2). The right ventricular hypertrophy indicated that rats exposed to hypoxia had developed pulmonary hypertension (PH rats). The retarded growth, polycythaemia and vascular hypertrophy are characteristic features of the development of hypoxic pulmonary hypertension.

The endothelium was functional in pulmonary arteries from both control and pulmonary hypertensive rats. Relaxant responses to ACh were (percentage reversal of induced contraction): main pulmonary artery, control rats 45±3.8 ($n=23$); PH rats (1 week hypoxia) 59±3.3 ($n=26$); PH rats (4 weeks hypoxia), 28±2.5 ($n=4$); intralobar pulmonary artery control rats 66±4.0 ($n=4$); PH rats (4 weeks hypoxia) 49±9.5 ($n=4$).

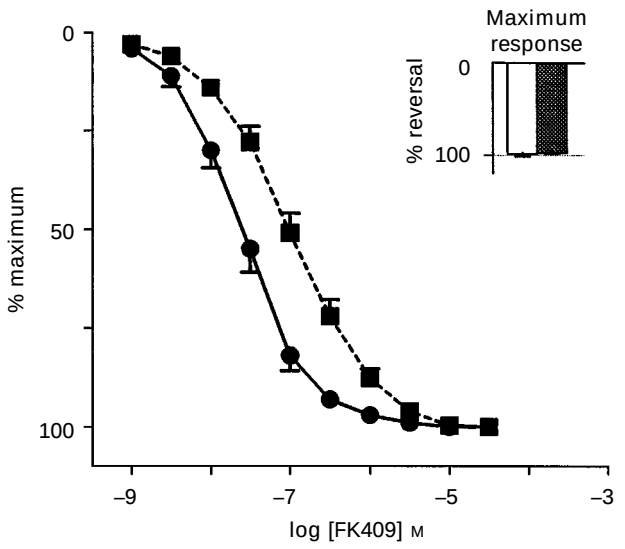

Figure 1 Mean concentration-response (relaxation) curves to FK409 on rat main pulmonary artery precontracted with noradrenaline (0.1 μM). Data under control conditions (●, $n=6$) and in the presence of 10 μM methylene blue (■, $n=3$) are shown. Mean responses are expressed as a percentage of the maximum relaxation to FK409. Inset: mean maximum relaxations to FK409, expressed as % reversal of the noradrenaline contraction, are represented (control conditions, open column; methylene blue, stippled column). The s.e.mean of responses are shown by the vertical lines.

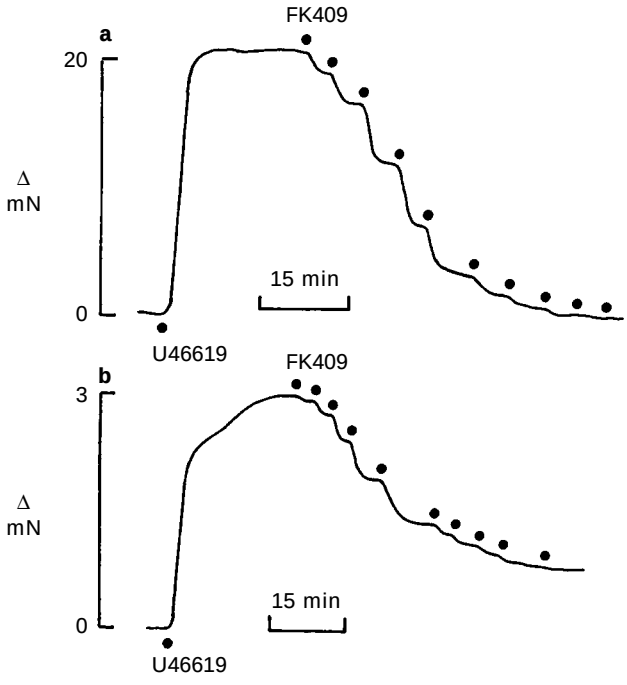

Figure 2 Experimental traces showing cumulative concentration-response (relaxation) curves to FK409 on U46619-contracted preparations of main pulmonary artery (a) and intralobar pulmonary artery (b) taken from control rats. Preparations were contracted with U46619 (30 nM on main pulmonary artery and 300 nM on intralobar pulmonary artery). The concentration range for FK409 was 1 nM to 30 μM (3 fold increments). The time scales represent 15 min. Note that FK409 completely reversed the contraction on main pulmonary artery but only partially reversed the contraction on intralobar pulmonary artery.

Table 1 Potency (negative log EC_{50}) of FK409 on main and intralobar pulmonary artery from control rats

Spasmogen	*Special conditions*	*FK409 (negative log EC_{50})* *Main pulmonary artery*	*Intralobar pulmonary artery*
Noradrenaline	–	7.62 ± 0.09 (6)	ND
Noradrenaline	Methylene blue	7.01 ± 0.11* (3)	ND
Noradrenaline	No endothelium	7.44 ± 0.17 (3)	ND
U46619	–	7.63 + 0.14[a] (4)	7.32 ± 0.06[a] (4)

Data shown are means ± s.e.mean of number of rats given in parentheses (*n*). [a]Data were obtained in paired preparations of main and intralobar pulmonary artery from the same rats. ND = not determined. *Value significantly less than the control value in the absence of methylene blue $0.05 > P > 0.01$ (one way ANOVA and Dunnet's *post hoc* test).

Table 2 Effects of exposure of rats to 10% oxygen for 1 or 4 weeks to induce pulmonary hypertension (PH rats)

	Control rats ($n = 23$)	*PH rats 1 week*[a] ($n = 26$)	*PH rats 4 weeks*[b] ($n = 6$)
Body weight of rats when 7–8 weeks old (g)	308 ± 8.1	255 ± 3.2##	252 ± 10.4##
Haematocrit (%)	44 ± 0.8	60 ± 0.8**	74 ± 3.3**
RV/(LV + S) (g g^{-1})	0.27 ± 0.01	0.40 ± 0.02**	0.63 ± 0.03**
RV/body weight (mg g^{-1})	0.56 ± 0.02	0.87 ± 0.03**	1.80 ± 0.15**
Cross-sectional area of main pulmonary artery (mm^2)[c]	0.66 ± 0.02	1.01 ± 0.04**	1.26 ± 0.07**[d]

Values are means ± s.e.mean; *n* = number of rats. [a]Rats exposed to hypoxia (10% O_2) for 1 week to induce pulmonary hypertension (PH). [b]Rats exposed to hypoxia (10% O_2) for 4 weeks to induce pulmonary hypertension (PH). [c]For definition of cross-sectional area see Methods. [d]$n = 4$. ##Value significantly less than corresponding value in control rats $0.01 > P > 0.001$. **Value significantly greater than corresponding value in control rats $0.01 > P > 0.001$ (one-way ANOVA and Dunnet's *post hoc* test).

Vasorelaxant effects of FK409 on pulmonary arteries from pulmonary hypertensive rats

Main pulmonary arteries from rats exposed to hypoxia for 1 week had inherent contractile tone. In these arteries, FK409 reversed not only the noradrenaline-induced tone but also the inherent tone; consequently maximum relaxation to FK409 was greater than 100% reversal of noradrenaline (Figure 3). In contrast, main pulmonary arteries from rats exposed to hypoxia for 4 weeks had little or no inherent tone and maximum relaxation to FK409 was 100%, as in control preparations (Figure 3). The potency of FK409 was significantly less than in control preparations whether rats were exposed to hypoxia for 1 week (Table 3; $P < 0.001$) or 4 weeks (negative log EC_{50} 7.08 ± 0.09, $n = 3$; $0.05 > P > 0.01$), but the reduction in potency was most pronounced after 1 week of hypoxia (Figure 3). After 4 weeks of hypoxia a reduction in potency was also seen in preparations of intralobar artery (negative log EC_{50} 6.23 ± 0.19, $n = 4$; $0.01 > P > 0.001$ when compared with the data in intralobar artery from control rats, Table 1); data were not obtained in intralobar artery from 1 week hypoxic rats.

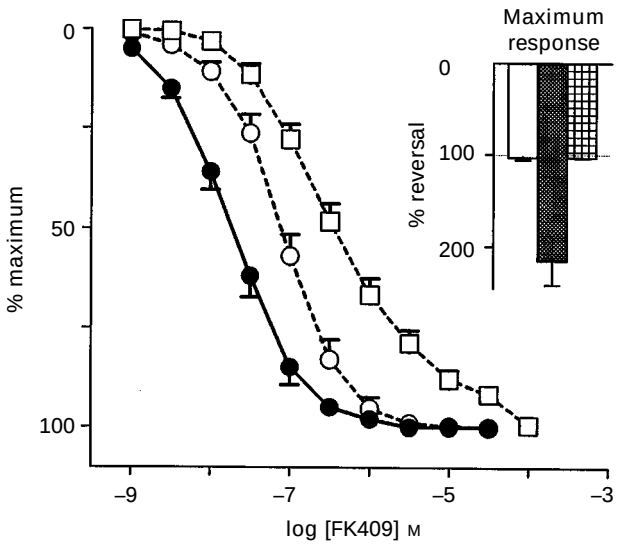

Figure 3 Mean concentration-response (relaxation) curves to FK409 on rat main pulmonary artery precontracted with noradrenaline (0.1 μM). Data were obtained in preparations from rats housed in room air (control rats ●, $n = 7$), or from rats in which pulmonary hypertension (PH) was induced by exposure to hypoxia (10% oxygen) for 1 week (1 week PH rats, □, $n = 8$), or 4 weeks (4 week PH rats, ○, $n = 3$). Mean responses are expressed as a percentage of the maximum relaxation to FK409. Inset: mean maximum relaxations to FK409, expressed as % reversal of noradrenaline contraction, are represented (control rats, open column; 1 week PH rats, stippled column; 4 week PH rats, cross-hatched column). The s.e.mean of all responses are shown by vertical lines.

Effects of in vitro *hypoxia, SOD, catalase and L-NAME on vasorelaxant effects of FK409 in main pulmonary artery from control and pulmonary hypertensive rats*

Experiments were carried out to investigate whether the reduction in potency of FK409 seen in arteries from pulmonary hypertensive rats might be due to an increase in one or more of the following reactive oxygen species: superoxide, hydrogen peroxide, hydroxyl or nitric oxide. Experiments in main pulmonary artery from control and pulmonary hypertensive rats (1 week of hypoxia) were therefore repeated (a) under conditions of reduced oxygen (95% nitrogen; *in vitro* hypoxia), (b) in the presence of SOD or catalase, either alone (to remove superoxide and hydrogen peroxide, respectively) or together (to remove superoxide, hydrogen peroxide and also hydroxyl) (Rubanyi & Vanhoutte, 1986) and (c) in the presence of L-NAME (to prevent production of nitric oxide).

Table 3 Potency (negative log EC_{50}) of FK409, under different *in vitro* experimental conditions, in main pulmonary artery from control and pulmonary hypertensive (PH) rats

In vitro conditions		FK409 (negative log EC_{50})	
Gas	*Drugs*	*Control rats*	*PH rats*[d]
Oxygen[a]	—	7.73±0.10 (7)	6.49±0.13*** (8)
Oxygen[a]	SOD and catalase[c]	7.67±0.12 (4)	7.12±0.03[##] (4)
Nitrogen[b]	—	7.47±0.07 (4)	7.16±0.02[##] (4)
Nitrogen[b]	SOD and catalase	7.56, 7.54 (2)	7.25±0.04[##] (3)

Data shown are means±s.e.mean of number of rats given in parentheses (*n*). [a]PSS bubbled with 95% O_2 plus 5% CO_2. [b]PSS bubbled with 95% N_2 plus 5% CO_2 (*in vitro* hypoxia). [c]SOD (150 u ml^{-1}) plus catalase (1200 u ml^{-1}). [d]Rats exposed to hypoxia (10% O_2) for 1 week to induce pulmonary hypertension (PH). ***Value significantly less than the corresponding value in control rats $P<0.001$. [##]Value significantly greater than the value in PH rats with oxygen present and no SOD or catalase $0.01>P>0.001$ (one-way ANOVA and Tukey Kramer *post hoc* test).

Treatment of the tissues with either SOD plus catalase or *in vitro* hypoxia (nitrogen) significantly increased the potency of FK409 in preparations from pulmonary hypertensive rats but had no effect on preparations from control rats (Table 3). Consequently, in the presence of either SOD plus catalase or nitrogen, there was no longer a significant difference in potency between the two groups of rats (Table 3). The combined effect of nitrogen and SOD plus catalase together was no different from the effect of either treatment alone (Table 3).

No effect on the potency of FK409 was seen, in either control or pulmonary hypertensive rats, with (i) SOD alone, (ii) catalase alone or (iii) L-NAME (data not shown).

Discussion

FK409 was found to be an effective pulmonary vasorelaxant in both main and intralobar pulmonary arteries from rats. The potency values for FK409 in these pulmonary artery preparations were greater than was found in rabbit aorta (Shibata *et al*., 1991), lower than in dog coronary arteries (Yamada *et al*., 1991; Kita *et al*., 1994) but comparable to values in a range of other systemic artery preparations from dogs (Ohtsuka *et al*., 1990). The observations that the effects of FK409 on rat main pulmonary artery were inhibited by methylene blue, but not by endothelial removal, are consistent with its classification as an NO donor drug.

FK409 was less effective on intralobar pulmonary arteries than on main pulmonary artery in that the maximum relaxation produced was significantly smaller and the potency slightly (2 fold) lower in the smaller arteries. These findings reflect data obtained with various other NO donor drugs, viz. nitroprusside, 3-morpholinosydnonimine (SIN-1) and KRN 2391, which all had smaller maximum responses and lower potencies on intralobar pulmonary arteries than on main pulmonary artery (Gambino *et al*., 1995; Wanstall, unpublished). In another study NO, itself, was less effective in relaxing resistance (small) compared with conduit (large) pulmonary arteries (Archer *et al*., 1996). Part of the vasorelaxant effect of NO may be via activation of a calcium-sensitive potassium channel (Kc_a), either directly (Bolotina *et al*., 1994) or via guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Archer *et al*., 1994), and recently it has been shown that K_{Ca} channels are more abundant in conduit pulmonary arteries than in resistance pulmonary arteries (Archer *et al*., 1996). These observations provide one possible explanation for the difference in potency of NO donors between large and small pulmonary arteries. It is interesting to note that in another vascular bed, i.e. the coronary vasculature, FK409 is likewise less potent on small vessels than on large vessels (Ohtsuka *et al*., 1990). It will be interesting to know whether the mechanism underlying this potency difference between vessels of different sizes is the same in the pulmonary and coronary circulations.

The experiments in arteries from pulmonary hypertensive rats showed that pulmonary hypertension was associated with a decrease in the potency, but not the maximum relaxation, of FK409 when compared with data obtained in control rats. This confirmed previous observations with other NO donors, viz. nitroprusside (Wanstall *et al*., 1992; Crawley *et al*., 1992; Rodman, 1992) and SIN-1 (Wanstall *et al*., 1995b), and suggests that it may be a feature of all drugs that act by producing NO. Previous studies have shown that this phenomenon is restricted to the pulmonary arteries, i.e. is not seen in aorta (Wanstall *et al*., 1992).

An interesting observation was that the reduction in potency seen for FK409 on main pulmonary artery was less pronounced after 4 weeks of hypoxia than after 1 week of hypoxia. In previous studies with a different NO donor (nitroprusside) the decrease in potency seen in pulmonary hypertensive rats was more pronounced after 2 weeks of hypoxia than after either 3 days (Wanstall *et al*., 1992) or 4 weeks (Wanstall; unpublished). These findings, together with the present data for FK409, suggest that in pulmonary hypertensive rats, the potency of NO donors may reach a low point after 1 to 2 weeks of hypoxia and, after that time, be partially restored. It is even possible that potency might return to control values if rats were exposed to hypoxia for periods of time greater than those used in studies to date, i.e. for more than 4 weeks. Experiments to investigate this possibility are currently in progress.

Two other differences between main pulmonary arteries from 1 week and 4 week hypoxic rats were noted. Arteries from 4 week hypoxic rats, like control arteries, had little or no inherent contractile tone and were quiescent. In contrast, arteries from 1 week hypoxic rats had substantial inherent tone and also displayed spontaneous contractile activity. The inherent tone has been shown previously in pulmonary arteries from rats exposed to hypoxia for either 1 week (Wanstall & Crilley, 1996) or 2 weeks (Wanstall *et al*., 1995a). It was postulated in earlier studies that the inherent tone may reflect a partially depolarized cell membrane (Wanstall *et al*., 1995a), possibly due to the closing of potassium channels (Smirnov *et al*., 1994). However this explanation now appears unlikely as membrane depolarization has been seen not only after 1 or 2 weeks of hypoxia, when there is inherent tone, but also after 4 weeks, when inherent tone is no longer apparent (Suzuki & Twarog, 1982). The spontaneous contractile activity is probably due to rapid oscillations in membrane potential (Twarog *et al*., 1988).

Although the reduction in potency of NO donors in pulmonary arteries from pulmonary hypertensive rats has now been described in a number of studies, the mechanism underlying this loss in potency remains unresolved. Data obtained by other authors suggest that it is unlikely to be due to either a reduced relaxant effect of cyclic GMP or an increase in metabolism of cyclic GMP by phosphodiesterases (Crawley *et al*., 1992). Hence it has been suggested that guanylate cyclase may be desensitized in pulmonary arteries from pulmonary hypertensive rats (Wanstall *et al*., 1992; Crawley *et al*., 1992). Any desensitization of guanylate cyclase could arguably be a consequence of augmented release of endothelial NO to compensate for the increase in pulmonary artery pressure (Crawley *et al*., 1992; Wanstall *et al*., 1992). Whilst the hypothesis that guanylate cyclase is desensitized is plausible, it has not yet been proven. Furthermore, even if guanylate cyclase were desensitized, this may not be the only factor responsible for the reduced potency of NO donors. In the present study an

alternative hypothesis was tested, namely that there may be increased production of reactive oxygen species in pulmonary arteries from pulmonary hypertensive rats.

The possible involvement of reactive oxygen species was considered for a number of reasons. Firstly, the half life of NO is influenced by the amounts of reactive oxygen species (especially superoxide, hydrogen peroxide and hydroxyl) in the surrounding environment as well as by the partial pressure of oxygen (P_{O_2}) (Kelm & Yosida, 1996). Secondly, reactive oxygen species have been implicated in another situation where the effects of NO donors are reduced (NO donor cross tolerance; Munzel *et al*., 1995). Thirdly, reactive oxygen species, especially superoxide, have been shown to be increased in another pathological situation that is associated, initially, with oxygen deprivation, i.e. ischaemia-reperfusion injury (Adkins & Taylor, 1990). FK409 was considered a suitable NO donor to test this hypothesis as it releases NO spontaneously in aqueous solution at neutral pH (Kita *et al*., 1994). SIN-1 is another NO donor that breaks down spontaneously in solution but, in the context of the present study, has the disadvantage that superoxide is, itself, a byproduct of the series of chemical reactions that lead to the production of NO from the molecule (Feelisch *et al*., 1989).

The data indicated that in control pulmonary arteries superoxide, hydrogen peroxide and hydroxyl were not present in sufficient quantities to affect the half-life of the NO produced from FK409, as neither SOD nor catalase, separately or together, had any effect on the potency of FK409. However, the results were different in arteries from pulmonary hypertensive rats. In these arteries a combination of SOD plus catalase did increase the potency of FK409. The same effect was observed when tissues were exposed to *in vitro* hypoxic conditions, i.e. were deprived of the oxygen necessary for the production of reactive oxygen species. Moreover, the effects of SOD and catalase and *in vitro* hypoxia together were not additive. Collectively these findings suggest that, in arteries from PH rats, reactive oxygen species (one or more types) were sufficiently abundant to reduce the half-life of NO and, hence, lower the potency of FK409. Superoxide alone appears not to be responsible for any reduction in the half-life of the NO as, surprisingly, SOD by itself was without effect. The data implicate hydrogen peroxide and superoxide together or, alternatively, hydroxyl radicals (Rubanyi & Vanhoutte, 1986). Hydroxyl could either interact with NO directly to produce nitrite (Kelm & Yoshida, 1996) or else have an inhibitory effect on NO-induced production of cyclic GMP (Marczin *et al*., 1992).

In summary, the results of this study have demonstrated that FK409 is a potent pulmonary vasorelaxant with properties consistent with its classification as an NO donor (viz. it is endothelium-independent but inhibited by methylene blue). Like other NO donors, it was slightly less effective on intralobar pulmonary arteries than on main pulmonary artery. Also like other NO donors, FK409 retained its ability to relax pulmonary arteries when preparations were taken from rats with chronic hypoxic pulmonary hypertension, although the potency of the drug was less than in control arteries. The reduction in potency was more pronounced in arteries from pulmonary hypertensive rats exposed to hypoxia for 1 week than after 4 weeks of hypoxia. The difference in potency between arteries from pulmonary hypertensive and control rats was not seen in the presence of SOD and catalase, or under hypoxic conditions *in vitro*. From this it is concluded that one or more reactive oxygen species may contribute to the reduction in potency of FK409 that occurs in pulmonary arteries from pulmonary hypertensive rats.

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References

ADKINS, W.K. & TAYLOR, A.E. (1990). Role of xanthine oxidase and neutrophils in ischaemia-reperfusion injury in rabbit lung. *J. Appl. Physiol*., **69,** 2012 – 2018.

ARCHER, S.L., HUANG, J.M.C., HAMPL, V., NELSON, D.P., SCHULTZ, P.J. & WEIR, E.K. (1994). Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A*., **91,** 7583 – 7587.

ARCHER, S.L., HUANG, J.M.C., REEVE, H.L., HAMPL, V., TOLAROVA, S., MICHELAKIS, E. & WEIR, E.K. (1996). Differential distribution of electrophysiologically distinct myocytes in conduit and resistance arteries determines their response to nitric oxide and hypoxia. *Circ. Res*., **78,** 431 – 442.

BOLOTINA, V.M., NAJIBI, S., PALACINO, J.J., PAGANO, P.J. & COHEN, R.A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature*, **368,** 850 – 853.

CELERMAJER, D.S., CULLEN, S. & DEANFIELD, J.E. (1993). Impairment of endothelium-dependent pulmonary artery relaxation in children with congenital heart disease and abnormal pulmonary hemodynamics. *Circulation*, **87,** 440 – 446.

CRAWLEY, D.E., ZHAO, L., GIEMBYCZ, M.A., LIU, S., BARNES, P.J., WINTER, R.J.D. & EVANS, T.W. (1992). Chronic hypoxia impairs soluble guanylyl cyclase-mediated pulmonary arterial relaxation in the rat. *Am. J. Physiol*., **263,** L325 – L332.

DINH-XUAN, A.T. (1993). Disorders of endothelium-dependent relaxation in pulmonary disease. *Circulation*, **87** (Suppl. V), V81 – 87.

DINH-XUAN, A.T., HIGENBOTTAM, T.W., CLELLAND, C.A., PEPKEZABA, J., CREMONA, G., BUTT, A.Y., LARGE, S.R., WELLS, F.C. & WALLWORK, J. (1991). Impairment of endothelium-dependent pulmonary-artery relaxation in chronic obstructive lung disease. *N. Engl. J. Med*., **324,** 1539 – 1547.

FEELISCH, M., OSTROWSKI, J. & NOACK, E. (1989). On the mechanism of NO release from sydnonimines. *J. Cardiovasc. Pharmacol*., **14** (Suppl. 11), S13 – S22.

GAMBINO, A., THOMAS, B.J. & WANSTALL, J.C. (1995). KRN2391 (a combined nitric oxide donor and potassium channel opener) on main and intralobar pulmonary arteries from rats. *Proc. Aust. Soc. Clin. Exp. Pharmacol. Toxicol*., **2,** 84.

GHODSI, F. & WILL, J.A. (1981). Changes in pulmonary structure and function induced by monocrotaline intoxication. *Am. J. Physiol*., **240,** H149 – H155.

HINO, M., IWAMI, M., OKAMOTO, M., YOSHIDA, K., HARUTA, H., OKUHARA, M., HOSODA, J., KOHSAKA, M., AOKI, H. & IMANAKA, H. (1989). FK409, a novel vasodilator isolated from the acid-treated fermentation broth of *Streptomyces griseosporeus*. 1. Taxonomy, fermentation, isolation and physico-chemical and biological characteristics. *J. Antibiotics*., **42,** 1578 – 1592.

ISONO, T., KOIBUCHI, Y., SATA, N., FURUICHI, A., NISHII, M., YAMAMOTO, T., MORI, J., KOHSAKA, M. & OHTSUKA, M. (1993). Vasorelaxant mechanism of the new vasodilator, FK409. *Eur. J. Pharmacol*., **246,** 205 – 212.

KAYE, J.A. & WANSTALL, J.C. (1995). Vasorelaxant properties of the nitric oxide donor, FK409, in pulmonary arteries from control and pulmonary hypertensive rats. *Proc. Aust. Soc. Clin. Exp. Pharmacol. Toxicol*., **2,** 85.

KELM, M. & YOSHIDA, K. (1996). Metabolic fate of nitric oxide and related N-oxides. In *Methods in Nitric Oxide Research*. ed. Feelisch, M. & Stamler, J.S. pp. 47 – 58, Chichester: Wiley.

KINSELLA, J.P., NEISH, S.R., SHAFFER, E. & ABMAN, S.H. (1992). Low-dose inhalational nitric oxide in persistent pulmonary hypertension of the newborn. *Lancet*, **340,** 819 – 820.

KITA, Y., HIRASAWA, Y., MAEDA, K., NISHIO, M. & YOSHIDA, K. (1994). Spontaneous nitric oxide release accounts for the potent pharmacological actions of FK409. *Eur. J. Pharmacol.*, **257,** 123–130.

LEFER, A.M. (1993). Why nitric oxide donors? *J Cardiovasc. Pharmacol.*, **22** (Suppl. 7), v–vi.

MARCZIN, N., RYAN, U.S. & CATRAVAS, J.D. (1992). Effects of oxidant stress on endothelium-derived relaxing factor-induced and nitrovasodilator-induced cGMP accumulation in vascular cells in culture. *Circ. Res.*, **70,** 326–240.

MARUYAMA, J. & MARUYAMA, K. (1994). Impaired nitric oxide-dependent responses and their recovery in hypertensive pulmonary arteries of rats. *Am. J. Physiol.*, **266,** H2476–H2488.

MUNZEL, T., SAYEGH, H., FREEMAN, B.A., TARPEY, M.M. & HARRISON, D.G. (1995). Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance. *J. Clin. Invest.*, **95,** 187–194.

MURPHY, R.A. (1980). Mechanics of vascular smooth muscle. In *Handbook of Physiology, the Cardiovascular System*. ed. Bohr, D.F., Somlyo, A.P. & Sparks, H.V. Sect. 2, Vol. 2, pp. 325–351. Bethesda, MD: American Physiological Society.

OHTSUKA, M., KOIBUCHI, Y., SAKAI, S., SATO, N., ISONO, T., ONO, T., MORI, J. & SHIBAYAMA, F. (1990). Cardiovascular activity of FK409, a new drug for ischaemic heart diseases, on dog in vitro and in vivo preparations. *Eur. J. Pharmacol.*, **183/4**, 1292–1293.

PEPKE-ZABA, J., HIGENBOTTAM, T.W., DINH-XUAN, A.T., STONE, D. & WALLWORK, J. (1991). Inhaled nitric oxide as a cause of selective pulmonary vasodilatation in pulmonary hypertension. *Lancet*, **338,** 1173–1174.

ROBERTS, J.D., POLANER, D.M., LANG, P. & ZAPOL, W.M. (1992). Inhaled nitric oxide in persistent pulmonary hypertension of the newborn. *Lancet*, **340,** 818–819.

RODMAN, D.M. (1992). Chronic hypoxia selectively augments rat pulmonary artery Ca^{++} and K^{+} channel-mediated relaxation. *Am. J. Physiol.*, **263,** L88–L94.

RUBANYI, G.M. & VANHOUTTE, P.M. (1986). Oxygen-derived free radicals, endothelium, and responsiveness of vascular smooth muscle. *Am. J. Physiol.*, **250,** H815–H821.

SHIBATA, S., SATAKE, N., SATA, N., MATSUO, M., KOIBUCHI, Y. & HESTER, R.K. (1991). Characteristics of the vasorelaxing action of (3E)-4-ethyl-2-hydroximino-5-nitro-3-hexamide FK409, a new vasodilator isolated from microbial sources, in isolated rabbit arteries. *J. Cardiovasc. Pharmacol.*, **17,** 508–518.

SMIRNOV, S.V., ROBERTSON, T.P., WARD, J.P.T. & AARONSON, P.I. (1994). Chronic hypoxia is associated with reduced delayed rectifier K^{+} current in rat pulmonary artery muscle cells. *Am. J. Physiol.*, **266,** H365–H370.

SUZUKI, H. & TWAROG, B.M. (1982). Membrane properties of smooth muscle cells in pulmonary hypertensive rats. *Am. J. Physiol.*, **242,** H907–H915.

TWAROG, B.M. (1988). Pathogenesis of pulmonary hypertension in the rat model. *Chest*, **93,** 100S–101S.

WANSTALL, J.C. (1994). *In vitro* hypoxia attenuates vasorelaxation by potassium channel opening drugs and nitroprusside in isolated pulmonary arteries from rats. *J. Pharmacol. Exp. Ther.*, **271,** 845–851.

WANSTALL, J.C. & CRILLEY, T.K. (1996). Relaxation of rat pulmonary artery by adrenomedullin involves endothelium-derived nitric oxide and is attenuated in hypoxic pulmonary hypertension. *Pharmacol. Commun.*, **7,** 349–357.

WANSTALL, J.C., HUGHES, I.E. & O'DONNELL, S.R. (1992). Reduced relaxant potency of nitroprusside on pulmonary artery preparations taken from rats during the development of hypoxic pulmonary hypertension. *Br. J. Pharmacol.*, **107,** 407–413.

WANSTALL, J.C., HUGHES, I.E. & O'DONNELL, S.R. (1995a). Evidence that nitric oxide from the endothelium attenuates inherent tone in isolated pulmonary arteries from rats with hypoxic pulmonary hypertension. *Br. J. Pharmacol.*, **114,** 109–114.

WANSTALL, J.C. & O'DONNELL, S.R. (1992). Responses to vasodilator drugs on pulmonary artery preparations from pulmonary hypertensive rats. *Br. J. Pharmacol.*, **105,** 152–158.

WANSTALL, J.C., THOMAS, B.J. & GAMBINO, A. (1995b). Vasorelaxation by nitric oxide donor drugs on pulmonary arteries from pulmonary hypertensive rats. *Br. J. Pharmacol.*, **116,** 61P.

WILLIAMSON, D.J., HAYWARD, C., ROGERS, P., WALLMAN, L.L., STURGESS, A.D., PENNY, R. & MACDONALD, P.S. (1996). Acute hemodynamic responses to inhaled nitric oxide in patients with limited scleroderma and isolated pulmonary hypertension. *Circulation*, **94,** 477–492.

YAMADA, H., YONEYAMA, F., SATOH, K. & TAIRA, N. (1991). Comparison of the effects of the novel vasodilator FK409 with those of nitroglycerin in isolated coronary artery of the dog. *Br. J. Pharmacol.*, **103,** 1713–1718.

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