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Direct gas measurements indicate that the novel cyclooxygenase inhibitor AZD3582 is an effective nitric oxide donor *in vivo*

[1]L. Christofer Adding, [1]Per Agvald, [2]Lars I. Andersson, [3]Bror Jonzon, [2]Janet Hoogstraate & *,[1,4]Lars E. Gustafsson

[1]Department of Physiology and Pharmacology, Karolinska Institute, SE-171 77 Stockholm, Sweden; [2]Research DMPK & Biomarkers, AstraZeneca R&D Södertälje, S-151 85 Södertälje, Sweden; [3]Experimental Medicine, AstraZeneca R&D Södertälje, S-151 85 Södertälje, Sweden and [4]Center for Allergy Research, Karolinska Institute, Stockholm, Sweden

1 AZD3582 [4-(nitrooxy)butyl-(2*S*)-2-(6-methoxy-2-naphthyl)propanoate] is a COX-inhibiting nitric oxide donor that inhibits COX-1 and COX-2. It is as effective as naproxen in models of pain and inflammation, but causes less gastroduodenal damage. Nitric oxide (NO) is generated from AZD3582 *in vitro*, and this study sought to show that the drug donates NO *in vivo*.

2 In anaesthetised male New Zealand white rabbits, the endogenous NO concentration in exhaled air was reduced by N^G-nitro-L-arginine methyl ester (L-NAME) (30 mg kg^{-1} i.v.) from 33.5±1.0 ppb (mean±s.e.m.; $n=6$ per group) to 3.0±1.0 ppb, while increasing blood pressure and reducing heart rate. AZD3582 (0.2, 0.6, 2.0 or 6.0 μmol kg^{-1} min^{-1}) given 30 min after L-NAME increased the concentration of NO in exhaled air ($P<0.05$), decreased blood pressure and increased heart rate in a dose-dependent manner *versus* L-NAME control values. The peak mean NO concentration obtained was 44±8.0 ppb.

3 In *in situ*-perfused rabbit lungs, L-NAME (185 μmol l^{-1}) reduced the NO concentration in exhaled air from 106±13 to 4.0±0.4 ppb ($n=5$). Addition of AZD3582 (6 μmol min^{-1}) to the perfusate produced an initial rapid increase in the NO concentration in exhaled air, followed by a sustained, but lower plateau. Infusion of L-NAME increased, and AZD3582 decreased, pulmonary arterial pressure.

4 In both anaesthetised rabbits and in the perfused lungs, brief periods of hypoxia increased NO concentrations generated by AZD3582.

5 We conclude that, in rabbits, AZD3582 donates NO *in vivo* with characteristics similar to those reported for nitroglycerin and isosorbide nitrates.
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Abbreviations: AUC, area under the curve; CINOD, COX-inhibiting nitric oxide donator; $ETCO_2$, end-tidal carbon dioxide; $FICO_2$, fraction of inspired CO_2; FIO_2, fraction of inspired O_2; GTN, glyceryl trinitrate; IP, insufflation pressure; LAP, left atrium pressure; L-NAME, N^G-nitro-L-arginine methyl ester; MAP, mean arterial pressure; mtALDH, mitochondrial aldehyde dehydrogenase; $PaCO_2$, carbon dioxide pressure in arterial blood; PaO_2, oxygen pressure in arterial blood; PAP, pulmonary artery pressure; PEEP, positive end-expiratory pressure; $PperfCO_2$, perfusion pressure of carbon dioxide; $PperfO_2$, perfusion pressure of oxygen; PVR, pulmonary vascular resistance

Introduction

Nonselective, nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit both cyclooxygenase (COX)-1 and -2 and are effective in managing pain and inflammation. However, their use is complicated by an increased risk of gastrointestinal bleeding and peptic ulcers (Soll *et al*., 1991; Cryer & Feldman, 1999). It is widely accepted that inhibition of endogenous prostaglandin production in the gastric mucosa plays a major role in NSAID gastrotoxicity, as these prostaglandins stimulate the formation of protective mucus and increase the production of bicarbonate (Wolfe *et al*., 1999). The relative contribution of the inhibition of COX-1 and COX-2 to gastrointestinal damage can be demonstrated by studies in rats. Selective inhibition of COX-1 or of COX-2 does not result in gastric damage in the rat; mucosal injury in the stomach is only seen when both isoforms are inhibited (Wallace *et al*., 2000; Gretzer *et al*., 2001). In the small intestine, inhibition of both COX-1 and COX-2 is also required before mucosal damage occurs (Tanaka *et al*., 2002).

The COX-inhibiting nitric oxide donor (CINOD) class of drugs was developed for the treatment of acute and chronic pain. CINODs have a multipathway mechanism of action that involves COX inhibition and NO donation (Wallace *et al*., 1994a, b; Davies *et al*., 1997; del Soldato *et al*., 1999). The rationale underlying the development of this class of drugs was that NO mediated many of the gastrointestinal processes that contribute to the protection of the gastric mucosa and exerts many of the same physiologically protective actions as the prostaglandins (Shanbhag *et al*., 1992). Thus, the donated

*Author for correspondence at: Department of Physiology and Pharmacology, Karolinska Institute, SE-17177 Stockholm, Sweden; E-mail: lars.gustafsson@fyfa.ki.se

NO may counter the detrimental effects of prostaglandin deficiency in the gastrointestinal tract that accompany COX-1 and COX-2 inhibition, while maintaining analgesic and anti-inflammatory efficacy from inhibition of both COX isoforms (del Soldato *et al*., 1999; Muscara & Wallace, 1999). The CINOD concept has been examined in a range of animal models with a number of chemical structures (Wallace *et al*., 1994a, b; Davies *et al*., 1997; del Soldato *et al*., 1999). AZD3582 (4-(nitrooxy)-butyl-(*S*)-(6-methoxy-2-naphthyl)-propanoate) is one such CINOD that has been shown to have gastrointestinal-protective properties in an animal model (Davies *et al*., 1997; Ojteg *et al*., 2002), and causes fewer erosions than naproxen in a short-term study in healthy volunteers (Hawkey *et al*., 2003). In this study, an anaesthetised rabbit model and an *in situ*-perfused rabbit lung model were used to examine the characteristics of NO generation from AZD3582 by monitoring the concentration of the gas in exhaled air. In the *in situ*-perfused rabbit lung model, haemoglobin is absent due to the perfusion with buffer. Haemoglobin scavenges NO and, therefore, will affect the NO measurements in the nonperfused situation (Rimar & Gillis, 1993; Wennmalm *et al*., 1993).

Methods

The local ethics committee approved the experiments.

Anaesthetised rabbits

Male New Zealand White rabbits were anaesthetised by injecting sodium pentobarbitone (50–60 mg kg^{-1}) into a marginal ear vein. Animals were placed on their backs on a heated operating table and a tracheotomy tube was inserted; they were then ventilated using a Harvard Apparatus rodent ventilator (model 638; South Natick, MA, U.S.A.). Air going to the ventilator was NO free; this was achieved by either filtering the air through a charcoal filter (150 × 12 cm) or by mixing commercially available N_2, CO_2 and O_2 (AGA, Lidingö, Sweden). The CO_2 and O_2 content of the air was accurately controlled with precision mass flow meters (Bronkhorst, Ruurlo, Netherlands). The fraction of inspired O_2 (FIO_2) could be adjusted in the range 0–100% and the fraction of inspired CO_2 ($FICO_2$) in the range 0–10%. The NO component was always below the level of detection (1.0 ppb). The concentrations of O_2 and CO_2 in inspired and exhaled air in the tracheal cannula were monitored continuously (Oscar-Oxy, Datex, Helsinki, Finland). The ventilation rate was 40 min^{-1} and the tidal volume was adjusted to keep end-tidal CO_2 at 5.0–5.5%; the minute ventilation was approximately 0.6 l min^{-1}. A positive end-expiratory pressure (PEEP) of 1–2 or 4–5 cm H_2O was provided by venting the exhaled gas under the surface of water in a beaker. The insufflation pressure was recorded continuously from the tracheal cannula with a pressure transducer (Statham, Hato Rey, Puerto Rico).

Once the animals were anaesthetised and the tracheal cannula in place, a polyethylene cannula was inserted into the left jugular vein and a continuous infusion was begun of glucose (27.5 g l^{-1}), dextran 70 (28 g l^{-1}), $NaHCO_3$ (7 g l^{-1}) and sodium pentobarbitone (4.8 g l^{-1}) at 5 ml kg^{-1} h^{-1} (STC-521 syringe pump, Terumo Corp., Tokyo, Japan). Experimental drugs were infused through a separate cannula, the tip of which was inserted into the other cannula. Blood pressure and heart rate were monitored continuously by means of a heparinised catheter in the right carotid artery connected to a pressure transducer (P23 AC, Statham Instruments, Hato Rey, Puerto Rico). Samples of arterial blood could also be taken from the right carotid artery, and blood gases were determined with an ABL 300 instrument (Radiometer, Copenhagen, Denmark). The animals' body temperatures were maintained at 38–39°C by means of a heated pad controlled through a thermostat (Wittman-Heraeus, Heidelberg, Germany).

Perfused in situ *lung experiments*

The rabbits were prepared as described above, after which the thorax was opened in the midline, and heparin (1000 IU kg^{-1} body weight, i.v.) was given. The PEEP was increased to 4–5 cmH_2O to prevent atelectasis when the thorax was opened. The common pulmonary artery was then cannulated via the right atrium and the lungs perfused *in situ* at 60 ml min^{-1} by means of a peristaltic pump (Sigmamotor, Middleport, NY, U.S.A.), draining perfusate from the system through catheters in the left atrium and left ventricle. The composition of the perfusate was as follows: NaCl, 125 mmol l^{-1}; KCl, 4.3 mmol l^{-1}; $CaCl_2$, 2.4 mmol l^{-1}; $MgCl_2$, 1.3 mmol l^{-1}; NaH_2PO_4, 1.2 mmol l^{-1}; glucose, 8.3 mmol l^{-1}; $NaHCO_3$, 35 mmol l^{-1}. Buffering was maintained by bubbling the solution with 7% CO_2 in N_2 and the solution was heated to 37°C. Pressures in the pulmonary artery and left atrium (PAP and LAP, respectively) were recorded continuously using Statham pressure transducers, which were used to calculate pulmonary vascular resistance: $PVR = (PAP–LAP)/Q$, where Q is the flow rate (60 ml min^{-1}).

When the perfusion was started, the $FICO_2$ was set to 5% and the composition of the exhaled air and acid–base status was determined. If necessary, the $FICO_2$ was adjusted in the range 4.8–5.3% so that the pH of the perfusate leaving the lungs was 7.35–7.45. This $FICO_2$ was then used for the rest of the experiment. After a 20–30-min stabilisation period (during which the lungs were perfused with at least 1 l of solution), the fluid leaving the lungs appeared clear and colourless, and the concentration of NO in the exhaled air, and pulmonary arterial and left atrial pressure were obtained.

Gas analysis

Respiratory gas was sampled continuously at 40 ml min^{-1} through a dehumidifying tube connected to the tracheal cannula. Sampling volume was less than 5% of the minute volume and gave stable NO concentrations during exhalation. NO concentration was determined by chemiluminescence in a breath-to-breath manner, as previously described (Agvald *et al*., 2002). The limit of detection was 1.0 ppb and the response time of the system ($T_{10–90}$) was 0.35 s. The detection system was calibrated using certified NO in nitrogen (AGA Specialgass, Lidingö, Sweden). A continuous recording was made of the concentration of NO in both inspired and exhaled air, together with insufflation pressure, end-tidal CO_2 and haemodynamic parameters (Grass model 7 Polygraph, Grass Instruments, Quincy, MA, U.S.A.).

Experimental design

Anaesthetised rabbits Rabbits were assigned randomly to one of the five dosage groups of six animals each (placebo group, $n=3$). Once the rabbits had stabilised for at least 30 min after the surgical procedures, each was given a dose of the NO-synthase inhibitor L-NAME (30 mg kg^{-1}). At 30 min after receiving L-NAME, animals were given the first of three consecutive infusions of AZD3582 at 40 min intervals. The first two infusions lasted 5 min, and the third 10 min. Responses were measured as the maximal NO concentration during the first 3 min of infusion compared with the concentration immediately before the infusion was started. During the third infusion of AZD3582, a brief (1.5 min) period of severe hypoxia ($FIO_2=0$) was imposed 4.5–5 min after the start of infusion. The effect of hypoxia was assessed as the change in NO concentration from immediately before the hypoxia began to the concentration after 1.5 min of hypoxia.

Perfused lungs Once the perfusate was clear and pressures had stabilised, L-NAME was infused at a concentration of 185 μmol l^{-1}. After 20 min, the infusion of AZD3582 was started (6 μmol min^{-1}, 100 μmol l^{-1}). After another 15 min, the lungs were exposed to four 5-min periods of hypoxia. The lung preparations were randomised to different levels of hypoxia: $FIO_2=5$, 2.5, 1 or 0%. Between periods of hypoxia, the preparations were allowed to stabilise at an FIO_2 of 21%. Gas analyses and acid–base status were determined before and at the end of each period of hypoxia.

Materials

L-NAME was purchased from Sigma Chemical Company, St Louis, MO, U.S.A.; heparin from Kabi Vitrum, Stockholm, Sweden; pentobarbitone from Apoteksbolaget, Stockholm, Sweden; dextran 70 (Macrodex®) from Pharmacia Infusions, Uppsala, Sweden; AZD3582 was provided as a ready-to-use formulation by AstraZeneca, Södertälje, Sweden.

Statistics

Values are presented in the figures as means ± s.e.m., and in tables as means ± s.d. ANOVA was used to assess the significance of differences between repeated measures, with *post hoc* testing using a *t*-test with Bonferroni correction (multiple comparisons with a single control) or Tukey's test (pairwise comparisons). The Kolmogorov–Smirnov test was used to check for normal distributions, and for non-normal distributions, Friedman's repeated measures ANOVA (on ranks) or Dunn's test (for multiple comparisons). Correlations between changes in exhaled NO and arterial pressure were calculated using Pearson's product moment correlation. Significant differences were deemed to exist when $P<0.05$ (two-tailed). Statistical calculations were carried out using SigmaStat 2.03 (Jandel Corporation, San Rafael, CA, U.S.A.).

Results

Anaesthetised rabbits

Blood gases and acid–base parameters were stable throughout the experiments, as were cardiovascular parameters in the absence of drug administrations (data not shown). Once the surgical procedures had been completed, the mean plateau concentration of NO measured breath-to-breath in exhaled air was 33.5 ± 1.0 ppb ($n=27$). L-NAME reduced the concentration of NO in exhaled air (25 min after L-NAME, the concentration was 3.0 ± 0.1 ppb; $n=27$). Infusion of AZD3582 caused a rapid increase in the NO concentration in exhaled air. The increases were reproducible and dose dependent (Figure 1).

At the two lower doses of AZD3582, the concentration of NO in exhaled air remained approximately constant for the whole of the 5-min infusion period. For the higher two concentrations, however, the NO concentration rose rapidly to a peak before falling. This was most marked for the highest dose used (6.0 μmol kg^{-1} min^{-1}). When the infusion was stopped, NO concentrations returned to baseline within 10–15 min (Figure 1). The magnitude of the NO response fell with repeated infusions of AZD3582, with the effect being most marked at the highest dose (Figure 2).

During the third infusion of AZD3582, brief (1.5 min), severe hypoxia ($FIO_2=0$) imposed 4.5–5 min after the start of the infusion caused the concentration of NO in exhaled air to rise. The concentration continued to rise for a short time even when oxygen was restored, and the peak increase in exhaled NO was particularly marked for the two highest doses of AZD3582 (Figure 3).

The infusion of L-NAME significantly increased systolic, diastolic and mean arterial blood pressure, with a concomitant and significant decrease in heart rate. Infusion of AZD3582 produced dose-dependent falls in arterial blood pressure and increases in heart rate (Figure 4). A correlation between NO concentration and fall in blood pressure in response to AZD3582 was found for increases in NO concentration of

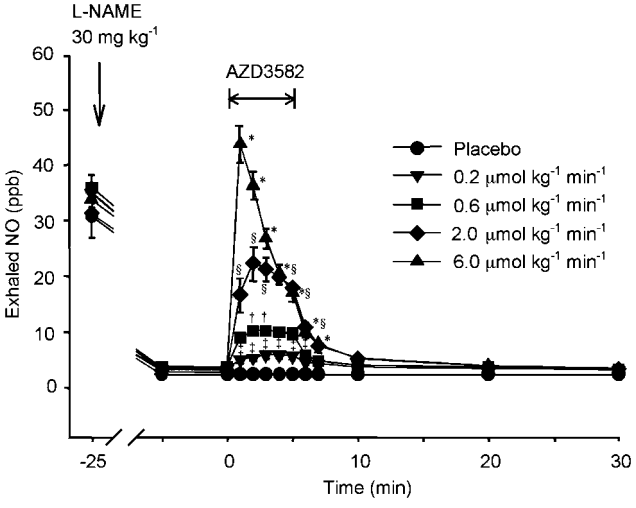

Figure 1 Effect of L-NAME and AZD3582 on the concentration of NO in exhaled air in pentobarbitone-anaesthetised rabbits. Means ± s.e.m. ($n=6$). ($P<0.05$ for placebo *versus* AZD3582 dose (μmol kg^{-1} min^{-1}): ‡0.2; †0.6; §2.0; *6.0.

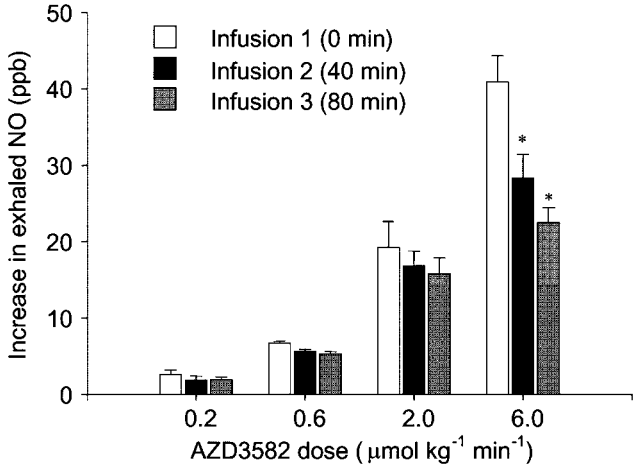

Figure 2 Effect of repeated infusions of AZD3582 at 40-min intervals on the generation of NO in anaesthetised rabbits. Columns represent the maximal increase (from baseline) in NO concentration in exhaled air during the first 3 min of AZD3582 infusion. Means ± s.e.m. ($n = 6$). (*$P < 0.05$ in comparison with infusion 1).

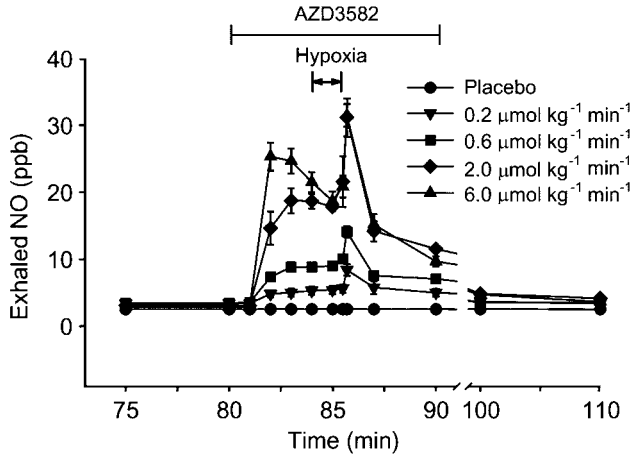

Figure 3 Effect of ventilatory hypoxia ($FIO_2 = 0\%$) on the concentration of NO in exhaled air in anaesthetised rabbits during the third of three consecutive infusions of AZD3582. Means ± s.e.m. ($n = 6$). ‡0.2; †0.6; §2.0; *6.0.

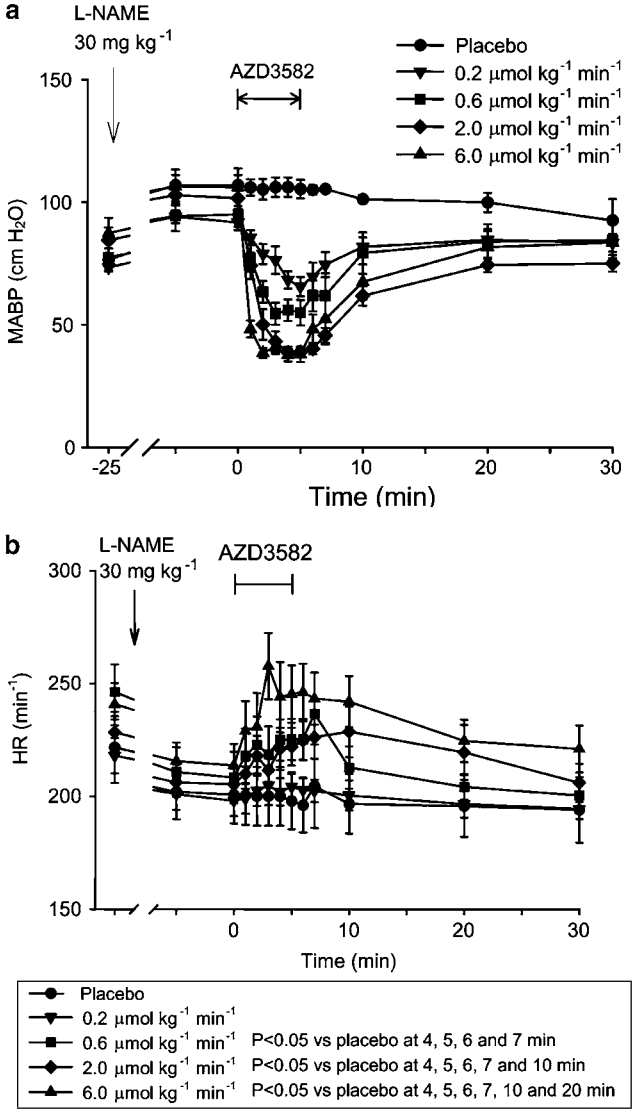

Figure 4 Effect of AZD3582 on (a) arterial blood pressure and (b) heart rate in anaesthetised rabbits. Means ± s.e.m.; $n = 6$ per group. For arterial blood pressure: $P < 0.05$ for all AZD3582 doses *versus* placebo at time points ⩾1 min except 0.2 μmol kg^{-1} min^{-1} at 1 and 20 min; 0.6 μmol kg^{-1} min^{-1} at 30 min; 2.0 μmol kg^{-1} min^{-1} at 20 and 30 min.

less than 12 ppb (Figure 5). Above this concentration, blood pressure did not change appreciably with increasing concentrations of NO, most likely due to a reached maximal vasodilatation effect of the infusion (see below and Figure 4a).

Dose–response curves for the fall in mean arterial blood pressure and NO release into the exhaled air (expressed either as area under the curve (AUC_{0-7}) (Figure 6a) or as peak concentration (Figure 6b)) were similar, although the blood pressure response showed signs of reaching a maximum at an infused dose of 6 μmol kg^{-1} min^{-1} of AZD3582, whereas the NO response curve was located further to the right and was still increasing with the maximal dose of AZD3582.

None of the respiratory parameters monitored (insufflation pressure (IP), end-tidal carbon dioxide ($ETCO_2$), PaO_2, $PaCO_2$, pH, base excess, haemoglobin, CO-haemoglobin and meth-haemoglobin) changed after L-NAME or in response to infusions of AZD3582.

In situ-*perfused rabbit lungs*

When perfusion began, the concentration of NO in the exhaled air rose rapidly and after the initial washout, the NO concentration stabilised at 106 ± 13 ppb ($n = 5$). Addition of L-NAME to the perfusate (185 μmol l^{-1}) markedly reduced the endogenous NO generation rate; 20 min after L-NAME was started, the NO concentration was 4.0 ± 0.4 ppb ($n = 5$). Against this background infusion of AZD3582 (6 μmol min^{-1}) produced a biphasic increase in NO concentration in exhaled air – an initial rapid peak, followed by a sustained, but lower plateau (Figure 7).

Addition of L-NAME also increased pulmonary vascular resistance, after which infusion of AZD3582 decreased pulmonary vascular resistance (Figure 7). Both these changes were mediated by changes in pulmonary arterial pressure rather than left atrial pressure. Neither L-NAME nor

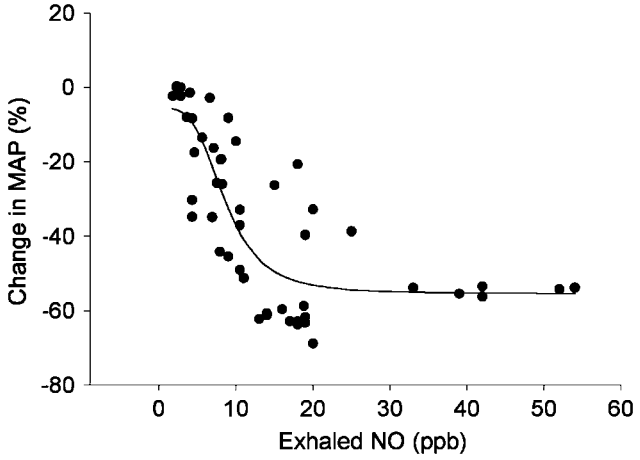

Figure 5 Correlation between the increase in exhaled NO and decrease in mean arterial pressure (MAP) in response to AZD3582. Mean ± s.e.m. ($n = 6$). The curve was plotted from data fit by non-linear regression.

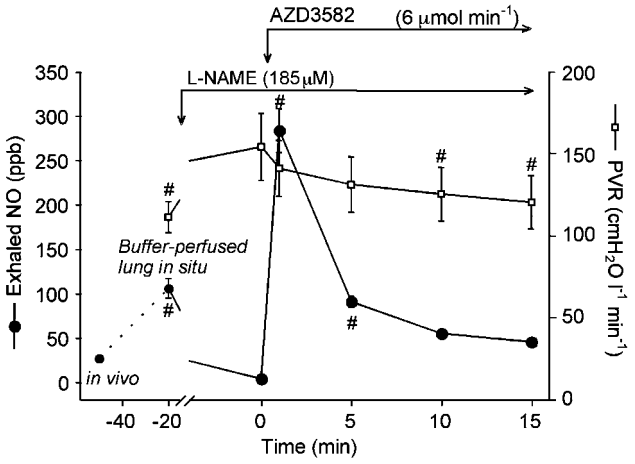

Figure 7 Effect of L-NAME and AZD3582 on the concentration of NO in exhaled air and on pulmonary vascular resistance in rabbit *in situ*-perfused lungs. Means ± s.e.m. ($n = 5$). *$P < 0.05$ *versus* value at time = 0 (Friedman's ANOVA on ranks with Dunn's *t*-test).

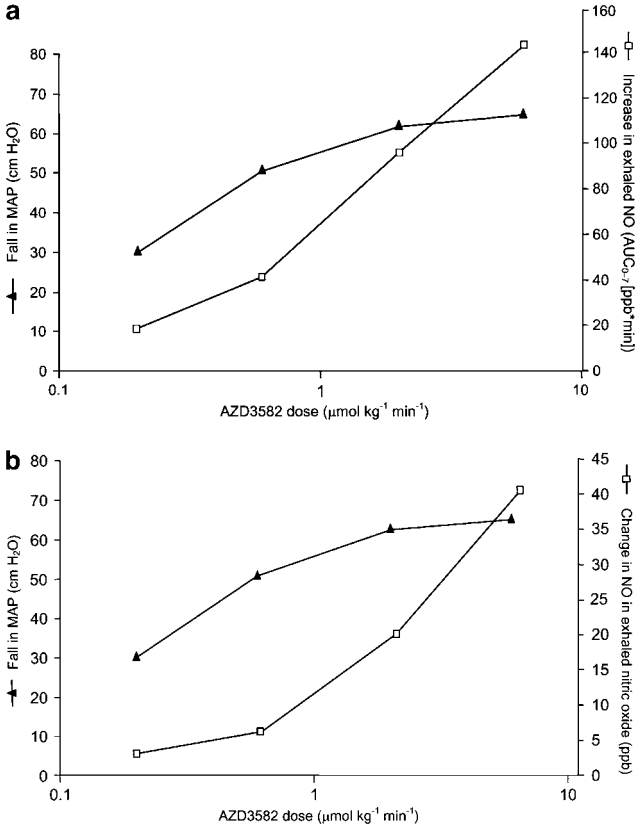

Figure 6 Dose–response curves for change in mean arterial pressure (MAP) and increase in exhaled NO in response to AZD3582. Values for MAP are calculated as the change from the value at the start of the infusion of AZD3582 to the value at the end of the infusion. Values for the change in NO are calculated as (a) the difference in AUC_{0-7} between the value after the infusion of placebo and the value after each dose of AZD3582, or (b) change in peak concentration of NO during the first 3 min of infusion of each dose of AZD3582.

AZD3582 changed any of the respiratory parameters monitored (IP, perfusion pressure of O_2 ($PperfO_2$), perfusion pressure of CO_2 ($PperfCO_2$) or pH).

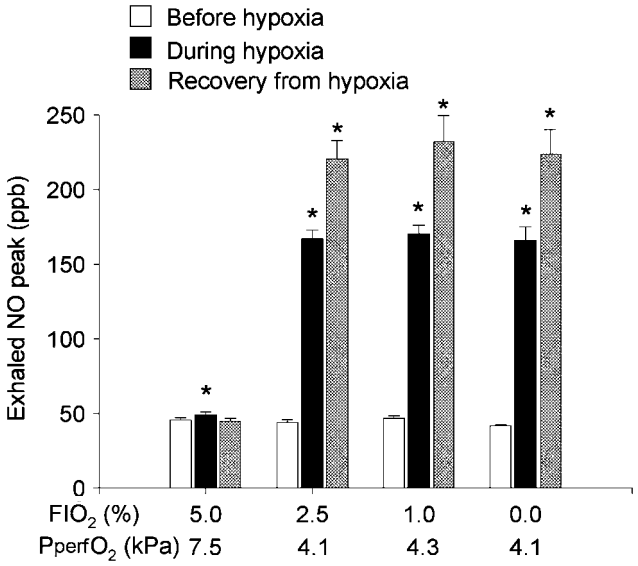

Figure 8 Effect of hypoxia on NO production in rabbit *in situ*-perfused lungs, during continuous infusion of AZD3582. NO concentration in exhaled air was measured just before the start of hypoxia (open bars), at the end of the 5-min hypoxia (solid bars) and during the first minute of normal oxygenation after the hypoxia (cross-hatched bars). *$P < 0.05$ *versus* value during normal oxygenation (open bars) (Student's *t*-test). Hypoxia during AZD3582 induced a minor (FIO_2 5%) to marked increase (at FIO_2 <5%) in exhaled NO. Note that at FIO_2 0%, some oxygen is still present, corresponding to severe hypoxic values in *in vivo* systems and illustrating that it is not possible to exclude fully the highly permeant oxygen molecule in biological models of this kind.

In response to 5-min periods of different degrees of hypoxia ($FIO_2 = 5$, 2.5, 1 or 0%), the amount of exhaled NO increased for FIO_2 5% and 2.5% and then plateaued at the higher degrees of hypoxia. The NO concentration rose steadily through each period of hypoxia and during the first minute after normal O_2 levels were restored (Figure 8).

Discussion

This study demonstrates that the CINOD, AZD3582, donates NO after intravenous administration to anaesthetised rabbits. A fraction of the donated NO reaches the pulmonary circulation from its site(s) of generation and is detectable in the exhaled air. Our experiments with *in situ*-perfused rabbit lungs confirm that AZD3582 donates NO and, furthermore, this also occurs in the absence of blood. It has also been recently demonstrated *in vitro* that, like glyceryl trinitrate (GTN), AZD3582 is a potent activator of the guanylyl cyclase/cGMP system, further evidence that AZD3582 is an NO donor (Berndt *et al*., 2004).

AZD3582 is considered to donate NO through a mechanism similar to that (or those) responsible for the generation of NO from organic nitrates such as GTN (Berndt *et al*., 2004). NO appears in exhaled air after the infusion of GTN in rabbits (Cederqvist *et al*., 1994; Persson *et al*., 1994), lambs (Husain *et al*., 1994) and humans (Marczin *et al*., 1997). NO is likely to be generated from organic nitrates through a variety of processes, including cytochrome P450 (Schröder *et al*., 1988; Bennett *et al*., 1992; Schröder, 1992; Mülsch *et al*., 1995; Yuan *et al*., 1997; Minamiyama *et al*., 1999), which is abundant in blood vessels (Mülsch *et al*., 1995; Yuan *et al*., 1997; Minamiyama *et al*., 1999). Other possible transformation pathways include glutathione-*S*-transferases (Taylor *et al*., 1989; Feelisch & Stamler, 1996) and xanthine oxidoreductase (Harrison, 2002).

The sites at which AZD3582 is metabolised to release NO have not been fully characterised. However, the release of NO from this CINOD is likely to involve lung tissues, as NO was generated from AZD3582 both in the *in situ*-perfused lung experiments and in the anaesthetised rabbit study. In our *in situ*-perfused lung experiments, infusion of AZD3582 produced a fall in pulmonary vascular resistance that appeared to be due to dilatation of pulmonary arterioles as left atrial pressure was unaffected. The fall in pulmonary vascular resistance is consistent with the beneficial effect of inhaled NO in pulmonary hypertension in humans (Barnes & Belvisi, 1993), and represents further evidence for NO donation. The most likely site for the generation of NO in this blood-free model is within the walls of the pulmonary arterial vessels, with subsequent activation of soluble guanylyl cyclase in arteriolar smooth muscle cells by the released NO. Among the cells that are exposed to infused drug in the buffer-perfused-lung model, endothelial and vascular smooth muscle cells can convert nitrovasodilators into NO (Mülsch *et al*., 1995; Yuan *et al*., 1997; Minamiyama *et al*., 1999). However, the contribution of alveolar and bronchial epithelial cells cannot be excluded considering the fact that the terminal respiratory units are supplied by blood of the pulmonary circulation. Therefore, bronchiolar/alveolar NO released into the airway lumen may be autoinhaled, with subsequent effects on pulmonary function, including vascular tone.

Notably, the concentration of AZD3582-derived NO in the exhaled air was considerably higher in the buffer-perfused-lung experiments (peak concentration was approximately 300 ppb) than in the anaesthetised rabbit experiments (peak approximately 45 ppb). The significance of this difference is uncertain because it is difficult to make a comparison between the doses – up to $6\,\mu mol\,kg^{-1}\,min^{-1}$ in anaesthetised rabbits *versus* $6\,\mu mol\,min^{-1}$ in perfused lungs. Furthermore, the concentration of exhaled NO in the anaesthetised rabbit experiments would be expected to be attenuated as a result of it being scavenged by haemoglobin (Rimar & Gillis, 1993; Wennmalm *et al*., 1993; Wink *et al*., 1996). Nevertheless, the exhaled concentrations of NO during moderately blood pressure lowering concentrations of AZD3582 in the presence of L-NAME are in the same order of magnitude as the endogenous NO concentrations before L-NAME.

The time courses of NO in exhaled air and fall in blood pressure in response to AZD3582 were similar. For the latter, plateaus were seen after the first few minutes of infusion of the two middle doses. At the lowest infused dose, the blood pressure fell throughout the whole infusion period, although the appearance of NO in exhaled air reached a plateau rapidly. At the highest dose, however, the blood pressure reached a plateau rapidly, whereas NO in exhaled air peaked rapidly and then fell despite continued infusion of AZD3582. As the rabbits were anaesthetised with pentobarbitone, the plateau phases in the blood pressure responses may not have been due to homeostatic responses mediated by hypothalamic control centres. However, cardiovascular reflexes were present, as demonstrated by the rapid fall in arterial blood pressure and increased heart rate at the highest AZD3582 dose. Since AZD3582 is known to improve cardiac function (Rossoni *et al*., 2004), such an effect might theoretically help in preventing deterioration of cardiac function during near-maximal or maximal vasodilation, possibly contributing to the levelling off of the blood pressure effects, but experiments beyond the scope of the present investigation would be needed to elucidate such an effect. Finally, the plateaus in the blood pressure response curves could mean that the maximal capacity for relaxation of blood vessels was reached at concentrations of NO lower than are generated *in vivo* from the highest doses of AZD3582 used. It is interesting to note that progressive vascular tolerance to GTN has been shown to develop during a maintained rate of formation of NO from GTN in tolerant tissues (Laursen *et al*., 1996). The dynamics of NO suggests that some step in the mechanism responsible for its generation from AZD3582 became saturated or that stores of an intermediate were exhausted.

The pattern of NO generation to exhaled breath and on systemic arterial pressure was very similar in AZD3582 (present data) when compared with studies on nitroglycerin and isosorbide di- and mononitrate (Agvald *et al*., 2002). The potency of AZD3582 in the NO generation (present data) was less than nitroglycerin and intermediate between those observed for isosorbide di- and mononitrate, (Agvald *et al*., 2002) and AZD3582 is therefore a moderately potent NO donor in this model. On a molar basis, it is approximately 10-fold less potent than nitroglycerin. AZD3582 has one nitrate ester group whereas nitroglycerin has three, but nitroglycerin mainly donates NO through loss of a first nitrate ester group *via* metabolism to glyceryl-1,2-dinitrate or to glyceryl-1,3-dinitrate, whereas the further metabolism to glyceryl mononitrate occurs with $t_{1/2}$ less than 1/10th of the rate in the initial step but from at least 10-fold higher concentrations of the metabolites (Jensen & Dahl, 1994). With this in mind, it could be argued that the NO donation from AZD3582 occurs with approximately five-fold less potency compared with nitroglycerin, considering the number of readily available groups for NO donation in nitroglycerin and its metabolites. After a therapeutically effective 750 mg AZD3582 oral b.i.d. dose, steady-state systemic plasma levels

of AZD3582 are 20 ± 17 nM in humans (Hawkey *et al*., 2003), although the presystemic levels of AZD3582 are expected to be much higher. Plasma concentrations of nitroglycerin are approximately 6 nM after sublingual administration of a single therapeutic dose (Jensen & Dahl, 1994). Thus, the therapeutic plasma concentrations of AZD3582 should be expected to donate pharmacologically active concentrations of NO provided AZD3582 is administered in a fashion that allows it to reach the circulation with its NO-donating group intact. The efficacy of AZD3582 as an NO donor was similar to that of nitroglycerin, when comparing the NO plateau during nitroglycerin infusion in the previous studies, and thus AZD3582 is a very efficacious NO donor.

As for nitroglycerin in our previous studies (Agvald *et al*., 2002), the highest doses employed for AZD3582 are well above therapeutic doses, and are employed in order to calculate dose–response relationships and to emphasise tachyphylaxis. In separate studies (P. Agvald *et al*., unpublished), we have been able to show that the tachyphylactic effects on blood pressure responses relate to tachyphylaxis also in exhaled NO concentrations during patch treatment with nitroglycerin at near-therapeutic concentrations in the rabbit. We therefore hypothesise that the present potency relationships apply also to therapeutic concentrations of the NO donors.

One important aspect of our results is that the apparent tachyphylaxis was particularly noticeable with the higher two doses of AZD3582. Since buffer was not recycled in the perfused-lung experiments, the possibility that some hypothetical mediator is released and that this inhibits NO production or opposes its effects on vascular smooth muscle can be ruled out. This tachyphylaxis is consistent with inactivation of an enzymic process. A mechanism for this is through depletion of stores of an intermediate. However, it is also known that cytochrome P450 enzymes are inhibited by NO or the peroxynitrite formed from NO and that NO may well be involved in the endogenous, physiological regulation of the activity of haem-containing enzymes, such as the cytochromes (Khatsenko *et al*., 1993; Alonso-Galicia *et al*., 1997; Oyekan & McGiff, 1998). This, therefore, suggests that responses to sustained infusion of AZD3582 should diminish as the released NO progressively inhibits cytochrome P450 gene (CYP) activity, thereby reducing the rate of its own production from AZD3582. Glutathione-*S*-transferases can potentially be inhibited by metabolites formed during their transformation of NO donors to NO (e.g. Lee & Fung, 2003), but this group of enzymes might be of less importance in NO donation in blood vessels as compared with in the liver (Salvemini *et al*., 1993). Another possibility is cofactor depletion during transformation of the organic nitrate to NO by xanthine oxidoreductase (Harrison, 2002). An alternative explanation for the observed tachyphylaxis may involve attenuation of mitochondrial aldehyde dehydrogenase (mtALDH) activity. Although the involvement of mtALDH in the generation of NO from AZD3582 is not known, Stamler (Chen *et al*., 2002) has hypothesised that GTN is biotransformed in mitochondria through a novel reductase activity of mtALDH and that the enzyme is inhibited in blood vessels made tolerant by GTN. Thus, the tachyphylaxis with AZD3582 could potentially arise from NO-induced inhibition of mtALDH.

The observed increases in NO in exhaled air with AZD3582 during hypoxia are to be expected. Agvald *et al*. (2002) showed that hypoxia dramatically increased the concentration of NO in exhaled air during infusion of GTN or isosorbide mononitrate, whereas there was a reduction in the rate of endogenous NO generation during hypoxic conditions. The rise in NO generation with AZD3582 during hypoxia occurred both in anaesthetised rabbits and in *in-situ* perfused rabbit lung preparations, and was therefore attributable, at least in part, to NO donation by AZD3582 within the lungs. Metabolism may increase the local concentration of reactive oxygen species that degrade released NO. Therefore, hypoxia would favour the preservation of released NO. In addition, NO is very labile and rapidly reacts with oxygen in the water phase with the exclusive formation of nitrite (NO_2^-). This reaction is 1000-fold faster than the formation of NO_2 in the gas phase or water phase (Wink *et al*., 1996). The reaction to NO_2 would be expected to lead to only negligible amounts of NO and oxygen being converted to NO_2^- and NO_3^-, which are formed either directly from the reaction of NO_2 with water or after formation of N_2O_4 and subsequent reaction with water. Furthermore, as this first step will not occur in the absence of O_2, the concentration of NO in the exhaled gas would be expected to rise.

NO is also broken down by reactive oxygen species, formed for example by spontaneous auto-oxidation of glucose (Beckman *et al*., 1996), a process also impaired during hypoxia (Beckman *et al*., 1996). The reaction with oxygen radical will result in the generation of peroxynitrite and subsequent hydrolysis to nitrite and nitrate or formation of nitrosothiols. Thus, in fact, NO may play a role in protecting cells against damage by reactive oxygen species (Wink *et al*., 1993; 1996). A third process is inactivation of NO to nitrate by interaction with haeme-containing proteins in the presence of oxygen (Wink *et al*., 1996). During hypoxia in the intact rabbit, more haemoglobin will be available as a carrier for NO, thus decreasing its breakdown and increasing its tissue distribution. However, hypoxia also increases NO levels from the perfused lung, arguing against the availability of haemoglobin as the only determinant of NO. Thus, several processes with critical dependence on oxygen availability may explain why NO levels increase during hypoxia. Since the function of cytochrome P450 is impaired during hypoxia, the rate at which NO is generated from a donor such as AZD3582 by cytochrome P450 should be expected to fall during hypoxia. Thus, it is obvious that the final concentration of NO will be dependent on a complex interaction of several systems.

We conclude, therefore, that in rabbits, AZD3582 is an effective NO donor *in vivo* and that the generation of NO from AZD3582 in this model does not depend on the presence of blood or major organ systems other than the lung. We also conclude that the rate at which NO appears in the exhaled air during infusion of AZD3582 is augmented during hypoxia, either because of increased generation of NO or because of inhibition of its breakdown. Thus, NO generation from AZD3582 will probably be most efficient in hypoxic/ischaemic inflammatory tissues. Additional experiments will be needed to further dissect the mechanisms responsible for the apparent tachyphylaxis and the effects of hypoxia.

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