British Journal of Pharmacology (1997) 122, 971–973 

SPECIAL REPORT

# Potency and kinetics of nitric oxide-mediated vascular smooth muscle relaxation determined with flash photolysis of ruthenium nitrosyl chlorides

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Flash photolysis of thermally stable, photolabile 'caged' precursors permits rapid and precise changes of ligand concentration at their site of action. This approach was used to determine the concentration-dependence and time course of NO-mediated relaxation of aortic smooth muscle, by use of two photolabile NO donors, trichloronitrosylruthenium ($Ru(NO)Cl_3$) and dipotassium pentachloronitrosyl-ruthenate ($K_2Ru(NO)Cl_5$). At concentrations up to 500 $\mu$M, both compounds were non-toxic before photolysis, and produced non-toxic by-products on photolysis. Photolytic release of NO produced relaxations of intact and endothelium-denuded aortic rings precontracted with noradrenaline (0.1–0.5 $\mu$M), with an $EC_{50}$ for NO-mediated relaxations of 10.5 nM and 13 nM, respectively. NO-mediated relaxations were reversibly blocked by 1 $\mu$M oxyhaemoglobin. The time course of NO-mediated relaxation comprised a delay of 3–7 s, followed by a sigmoidal decline in tension with peak rates that were strongly dependent on NO concentration.

**Keywords:** Nitric oxide; flash photolysis; relaxation; ruthenium nitrosyl chlorides

**Introduction** Nitric oxide (NO) has been implicated in many physiological and pathophysiological processes, but its role in regulating vascular smooth muscle tone and blood flow is particularly well established (Umans & Levi, 1995). Studies of the actions of NO in complex biological preparations are hampered by its high reactivity, and short half-life (0.1–6 s; Griffith *et al*., 1987; Kelm *et al*., 1988), and by uncertainties about the kinetics and extent of NO liberation from thermally unstable NO donors such as sodium nitroprusside. Problems associated with diffusional delays, particularly for unstable ligands such as NO, can be overcome by flash photolysis (see McCray & Trentham, 1989). In this method stable, biologically inactive precursors are equilibrated within the preparation, before rapid release of the active ligand at or close to its side of action by a brief pulse of near-u.v. light. Here, we describe the use of two water soluble, thermally stable and inexpensive NO-donors, trichloronitrosylruthenium ($Ru(NO)Cl_3$) and dipotassium pentachloronitrosylruthenate ($K_2Ru(NO)Cl_5$), with well-defined photochemistry (Bettache *et al*., 1996), to quantify the action of NO in the rabbit isolated aortic ring. Preliminary accounts of this work have been given (Carter *et al*., 1993; Bettache *et al*., 1996).

**Methods** Rabbits (male half-lop, 600–800 g) were killed by cervical dislocation and exsanguination. The thoracic aorta was removed and placed in oxygenated physiological saline solution (PSS) (mM: NaCl 145, KCl 5.6, $NaHCO_3$ 0.8, $MgSO_4$ 1, $CaCl_2$ 1.8 and glucose 11, gassed with 95% $O_2$/5%$CO_2$). The aorta was cut into rings ~4–5 mm in length, and mounted for isotonic measurements in a 10 ml organ bath. The organ bath incorporated a quartz window (0.15 mm thick) allowing exposure of the suspended aortic ring to near-u.v. light (300–350 nm) from a xenon arc flash lamp. In some experiments the endothelium was removed by gently rubbing the lumen of the aortic ring with tissue paper. Aortic rings were equilibrated for 60 min at 23–27°C. From the noradrenaline dose-response curve a sub-maximal concentration of noradrenaline was chosen (0.1–0.5 $\mu$M) to contract the tissue ring. Endothelium-dependent relaxations were evoked by cumulative addition of acetylcholine (ACh; 5–1000 nM) to the organ bath. Caged NO, diluted in noradrenaline-containing PSS, was added to the bath by means of a Hamilton syringe, and removed by perfusion of the bath with PSS which contained noradrenaline but no caged-NO. Photolysis of caged NO was with a 1 ms pulse of near u.v. light (300–350 nm selected with a UG11 filter) from a xenon arc flash lamp (Rapp & Guth, 1988). Calibration of the extent of NO released by a pulse of near u.v. light was determined by measuring the extent of photolysis of caged adenosine 5′-triphosphate (ATP) in a quartz cuvette placed at the position occupied by the aortic ring in the experiment, as previously described (Bettache *et al*., 1996). Briefly, the proportion of caged NO converted during a 1 ms flash was calculated from the proportion of caged ATP photolysed (determined by anion exchange high performance liquid chromatography (h.p.l.c.) multiplied by the ratio of the $Q_p$ values of caged NO and caged ATP ($Q_p = 0.06$; $K_2Ru(NO)Cl_5$, 0.012; $Ru(NO)Cl_3$, 0.63; caged ATP; Bettache *et al*., 1996). Oxyhaemoglobin was prepared from bovine haemoglobin (Sigma) as previously described (Bettache *et al*., 1996). $Ru(NO)Cl_3$ and $K_2Ru(NO)Cl_5$ were from Alfa, Johnson Matthey plc (Orchard Road, Royston, Hertfordshire, SG8 5HE). Other chemicals were Analar grade from BDH.

**Results** Aortic rings precontracted with noradrenaline were relaxed by acetylcholine (5–1000 nM) with an $EC_{50}$ for inhibition of noradrenaline-induced tone of 58 nM (see Figure 1b, $n = 7$ animals). Removal of the endothelium abolished relaxations to acetylcholine ($n = 3$, data not shown). Exposure of intact or endothelium-denuded aortic rings to a pulse of near-u.v. light produced either no response, a small transient relaxation (predominantly in intact rings) or a small transient increase in tone (predominantly in endothelium denuded rings). Exposure for up to 30 min to either $Ru(NO)Cl_3$ or $K_2Ru(NO)Cl_5 \leqslant 500$ $\mu$M produced no change in the absence of a pulse of near u.v.

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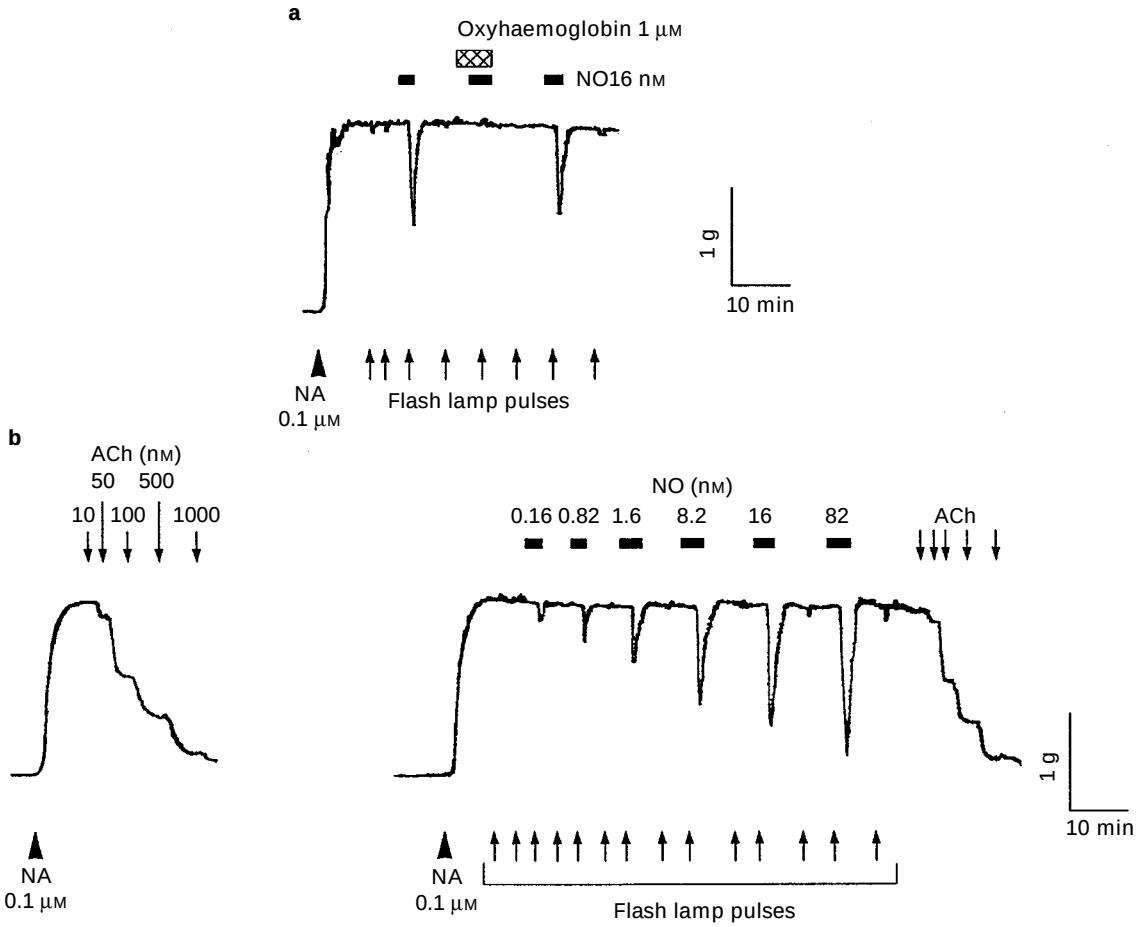


**Figure 1** (a) Relaxation of noradrenaline (NA)-precontracted endothelium-intact rabbit aortic ring by 16 nM NO photo-released from 2 $\mu$M $K_2Ru(NO)Cl_5$ applied briefly to the bath (solid bars), in the absence and presence (hatched bar) of 1 $\mu$M oxyhaemoglobin. (b) Relaxations of endothelium-intact aortic rings produced by cumulative additions of acetylcholine (ACh; left and end of right hand records) and 0.16–82 nM NO, photo-released from $Ru(NO)Cl_3$ (0.5–50 $\mu$M) applied briefly to the bath (solid bars). Arrows indicate times where a 1 ms pulse of near-u.v. light was applied to the tissue ring, either in the absence or presence of $Ru(NO)CL_3$.

light. Exposure to solutions of caged NO fully photolysed by prior u.v. irradiation produced no change in tone either with or without a subsequent pulse of near-u.v. light. In the presence of caged NO, a pulse of near-u.v. light produced a reduction in tone that recovered over 3–7 min to pre-flash levels (see Figure 1). Relaxations were reversibly blocked by addition of 1 $\mu$M oxyhaemoglobin (Figure 1a, $n=3$) showing that they were due to NO. Photorelease of NO over the range 0.08–4019 nM, from either $Ru(NO)Cl_3$ (Figure 1b and Figure 2b) or $K_2Ru(NO)Cl_5$ (Figure 2), produced concentration-dependent relaxation of noradrenaline-precontracted intact aortic tissue rings. At very high NO concentrations (>400 nM) relaxations equalled or slightly exceeded the increased tone produced by noradrenaline (see Figure 2b). Neither compound affected subsequent relaxations produced by ACh (data not shown). The time course of relaxation mediated by photoreleased NO in intact aorta is shown in Figure 2a. At high concentrations (410 nM NO) the response had a delay of 4.7 $\pm$ 0.5 s (mean $\pm$ s.e.mean, $n=5$), followed by a sigmoidal decline to minimum tension with a mean half-time of 23.8 $\pm$ 2.0 s, producing 92 $\pm$ 11% inhibition of the noradrenaline-induced tone. The delay showed only slight dependence on the NO concentration (Figure 2c), the peak rate of relaxation increased strongly at high NO concentrations, reaching a maximum at >400 nM NO (Figure 2d). The $EC_{50}$ value for NO-mediated inhibition of noradrenaline-induced tone in intact aortic rings was 10.5 nM (Figure 2b), and for endothelium-denuded rings was 13 nM (data not shown).

**Discussion** The results described here demonstrate that the rapid release of NO by flash photolysis of $Ru(NO)Cl_3$ or $K_2Ru(NO)Cl_5$ can be usefully applied to study the action of NO in isolated vascular tissue, yielding quantitative and kinetic information hard to obtain by other means. Photolysis of nitrosylruthenates produced rapid (<1 ms) release of known concentrations of NO at the endothelial or smooth muscle cell membrane. Following NO release there was a clear delay before relaxation, which cannot be explained by diffusional delays, but rather suggests a slow step or steps in the signal cascade initiated by NO. The rate of relaxation showed a strong dependence on NO concentration and reflects the net contribution of the signalling pathways linked to myosin light chain kinase (MLCK) and phosphatase (MLCP) activity. The rate limiting step in relaxation of tonic smooth muscle is likely to be the slow rate of dissociation of MgADP from actin-myosin crossbridges (0.033 $s^{-1}$; Khromov *et al.*, 1995), following dephosphorylation of the myosin light chain.

The $EC_{50}$ values for NO-mediated relaxation in intact and endothelium-denuded aortic rings were similar, 10.5 and 13 nM, respectively, and are in the same low nM range found to be effective by other approaches (Hutchinson *et al.*, 1987; Flitney *et al.*, 1996). The slightly higher value in endothelial denuded rings may reflect some damage to the smooth muscle layer as a result of removal of the endothelium. The approach described here allows the detailed investigation of the kinetics and concentration-dependence of the effect of NO in tissues where the instability of NO

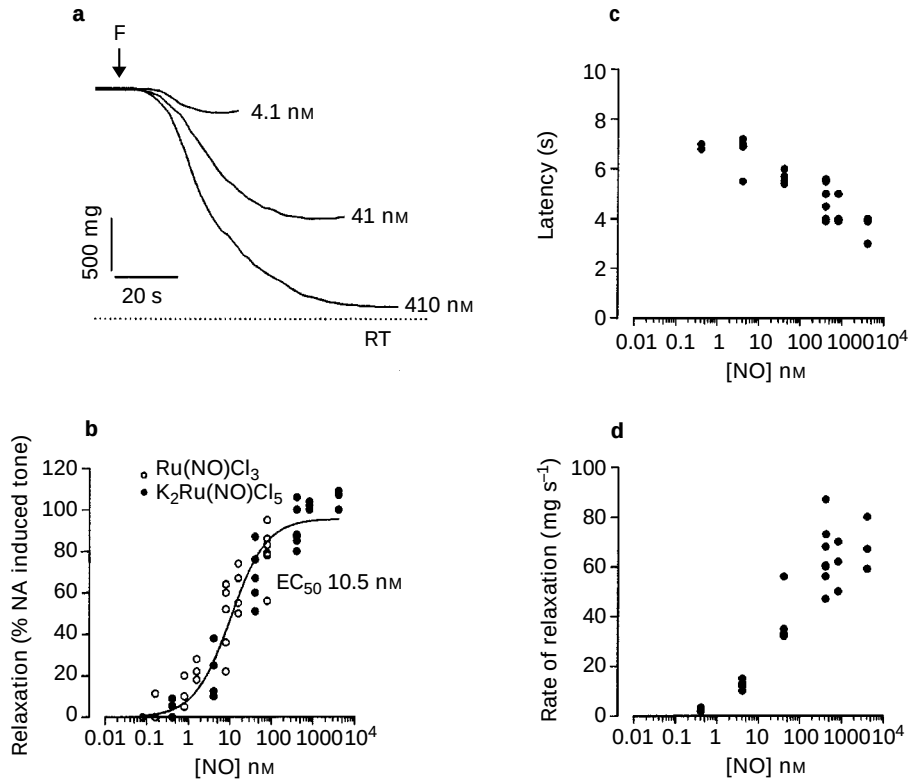


**Figure 2** The time course (a) and concentration-dependence (b) of NO-mediated relaxation produced by photolysis of $K_2Ru(NO)CL_5$ (a and b) and $Ru(NO)CL_3$ (b). Collected data for the latency (c) and rate of relaxation of (d) produced by NO, photoreleased from $K_2Ru(NO)CL_5$. RT; resting tone before noradrenaline addition. F; indicates point at which 1 ms pulse of near-u.v. light was applied to the tissue ring.

prevents quantitative experiments, and helps to identify the contribution of specific signalling pathways underlying the action of NO.

Supported by the Medical Research Council and British Heart Foundation. T.D.C. holds the British Heart Foundation Gerry Turner Intermediate Research Fellowship.

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*(Received July 31, 1997*
*Accepted September 16, 1997)*