Immunostaining and flow-cytometry analysis

Cells were smeared on glass slides by cytocentrifugation, stained as described[26] and analysed by epifluorescence microscopy. Alternatively, cells were fixed in 2% paraformaldehyde, permeabilized in 0.05% Triton, incubated with anti-GATA-1 or control antibody, stained with phycoerythrin-labelled secondary antibodies and analysed by flow cytometry. Data were expressed as mean fluorescence index ratio between anti-GATA-1 and control antibody values.

Expression of caspases in bacteria and GATA-1 cleavage assay

Complementary DNAs for human caspases were subcloned in bacterial expression vectors of the pET21 series in frame with amino-terminal T7 tag and a carboxy-terminal six-histidine tag, as described[27]. Proteins were expressed in BL21 (DE3) bacteria, purified on a Ni^{2+} affinity resin, assayed for efficient activity on specific fluorogenic peptides, and used for *in vitro* cleavage assay. GATA-1 translation reaction (1 μl) was incubated with 2 μl purified caspases in ICE buffer (25 mM Hepes, 1 mM EDTA, 5 mM DTT, 0.1% CHAPS, pH 7.5) in a final volume of 10 μl. The reaction was incubated for 1 hour at 37 °C and analysed by Tricine-SDS/PAGE and autoradiography.

Immunoblot and caspase activation assay

For detection of actin and caspases, cells were lysed in 1% NP40 lysis buffer in the presence of 1 mM phenylmethylsulphonyl fluoride and 2 μg ml^{-1} each of aprotinin, leupeptin and pepstatin. The cell extracts for detection of GATA-1, GATA-2 and lamin B were made with 2% SDS lysis buffer. Proteins were analysed by standard immunoblot procedure. Caspase activity was evaluated by DEVD cleavage in a fluorogenic assay as described[2].

Mutagenesis and *in vitro* transcription or translation

Potential aspartate processing sites of GATA-1 and the catalytic cysteine of caspase-8 were mutated to glutamate and alanine by site-directed mutagenesis, using overlapping polymerase chain reaction (PCR) mutagenic oligonucleotides to produce caspase-resistant GATA-1 and a dominant-negative mutant of caspase-8 (ref. 28). Wild-type and mutated cDNAs were transcribed *in vitro* and translated in the presence of [^{35}S]methionine.

Production of retroviral particles and infection of haematopoietic progenitors

FLAME-1, CrmA, C8DN, wild-type and mutated GATA-1 cDNAs were cloned into the PINCO vector[19]. Culture supernatants containing viral particles were collected at 48 h after the amphotropic packaging cell-line transfection, and $CD34^+$ cells exposed to cycling growth factors were infected[19]. GFP-positive cells were sorted 24 h after the last infection cycle using a FACS VANTAGE cell sorter (Becton Dickinson)[19].

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Cytochrome P450 2C is an EDHF synthase in coronary arteries

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In most arterial beds a significant endothelium-dependent dilation to various stimuli persists even after inhibition of nitric oxide synthase and cyclo-oxygenase. This dilator response is preceded by an endothelium-dependent hyperpolarization of vascular smooth muscle cells, which is sensitive to a combination of the calcium-dependent potassium-channel inhibitors charybdotoxin and apamin, and is assumed to be mediated by an unidentified endothelium-derived hyperpolarizing factor (EDHF)[1,2]. Here we show that the induction of cytochrome P450 (CYP) 2C8/34 in native porcine coronary artery endothelial cells by β-naphthoflavone enhances the formation of 11,12-epoxyeicosatrienoic acid, as well as EDHF-mediated hyperpolarization and relaxation. Transfection of coronary arteries with CYP 2C8/34 antisense oligonucleotides results in decreased levels of CYP 2C and attenuates EDHF-mediated vascular responses. Thus, a CYP-epoxygenase product is an essential component of EDHF-mediated relaxation in the porcine coronary artery, and CYP 2C8/34 fulfils the criteria for the coronary EDHF synthase.

The term EDHF is likely to comprise several factors, as the pharmacological characteristics of the nitric oxide synthase/cyclo-oxygenase (NOS/COX)-inhibitor-insensitive, endothelium-dependent hyperpolarization in different arteries and species are clearly distinct[3]. Although the EDHF that mediates relaxation in many vascular beds remains to be identified, there is indirect evidence that a CYP-epoxygenase-derived product mediates NOS/COX-independent relaxation in the human[4], porcine[5,6], bovine[5,7], canine[8,9] and rat[10,11] coronary circulations. Indeed, 11,12-, 14,15-, 8,9- and 5,6-epoxyeicosatrienoic acids (EETs) and their dihydroxyeicosatrienoic acid derivatives both hyperpolarize and relax coronary vascular

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smooth muscle cells[7,9,12]. Moreover, the P450 inducer β-naphthoflavone can enhance smooth muscle hyperpolarization elicited by the putative CYP-dependent EDHF[6]. A role for an endothelium-derived CYP product in agonist-induced vascular relaxation was proposed over a decade ago, on the basis of the observation that inhibition of phospholipase A_2 and CYP attenuates the transient endothelium-dependent relaxation of isolated canine arteries[1]. CYP inhibitors have since been shown to attenuate EDHF-mediated hyperpolarization and relaxation[4,11–13], but these compounds are not isoform-selective and can directly affect the activity of calcium-dependent potassium (K^+_{Ca}) channels[14,15]. Thus, an alternative, non-pharmacological approach is required to elucidate the role of a CYP enzyme in EDHF-mediated vascular responses.

To determine which CYP isoform could be implicated in the generation of EDHF-mediated responses we used polymerase chain reaction with reverse transcription (RT-PCR) to screen primary cultures of porcine aortic and human umbilical vein endothelial cells, as well as human coronary artery and lung microvascular endothelial cells. Of the CYP epoxygenases detected, 1A, 2C and 2J were expressed in porcine aortic and human umbilical vein endothelial cells, and 2C and 2J were also expressed in lung microvascular and coronary artery endothelial cells. Only the globally expressed CYP 2C family, and more specifically the human CYP 2C8 (homologous to the porcine CYP 2C34) fitted the criteria for an EDHF synthase, as its expression was consistently enhanced by β-naphthoflavone in all of the cultured endothelial cell types studied (not shown). Other CYP 2C isoforms of human origin are 2C9, 10, 17, 18 and 19. Of these, CYP 2C9 is an arachidonic acid epoxygenase which produces EETs and, although it is highly expressed in human liver, we could not identify this isoform by RT-PCR in any of the human endothelial cells tested. Neither CYP 2C17 (a splice variant of 2C18 and 2C19) nor 2C18 were detectable in any of the human endothelial cells and although CYP 2C19 was detectable, its expression was unaffected by β-naphthoflavone. Note, however, that CYP 2C18 and 2C19 are not reported to exhibit any epoxygenase activity[16].

To establish a functional link between the expression of CYP 2C8/34 and the production of EDHF, experiments were performed using isolated endothelium-intact porcine coronary arteries (PCA). Intraluminal incubation with β-naphthoflavone enhanced the expression of CYP 2C messenger RNA and protein in native coronary artery endothelial cells (Fig. 1a, b). This induction of CYP 2C resulted in a leftward shift in the EDHF-mediated concentration–relaxation curve to bradykinin ($EC_{50} = -6.18 \pm 0.33$ M in control versus -7.53 ± 0.40 M in β-naphthoflavone-treated rings, $P < 0.05$, $n = 4$; Fig. 1d), and potentiated the bradykinin-induced, EDHF-mediated hyperpolarization of PCA (Fig. 2a). No CYP 2C signal was detected in endothelium-denuded PCA. β-naphthoflavone did not affect the resting membrane potential of smooth

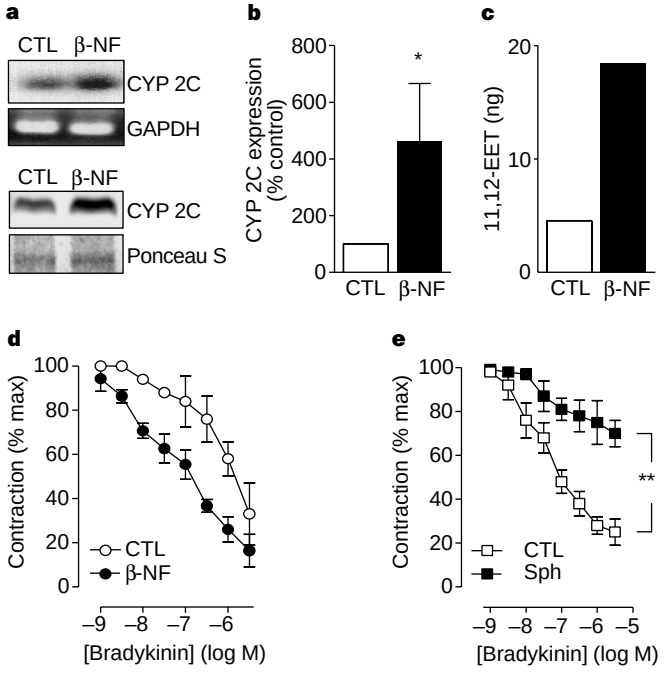

Figure 1 Increased expression of CYP 2C enhances EDHF-mediated relaxation of PCA and generation of 11,12-EET. PCA were perfused with culture medium containing either solvent (CTL; 48 h) or β-naphthoflavone (β-NF; 3 μM, 48 h). **a**, Total RNA, harvested by intraluminal incubation of PCA with guanidine isothiocyanate, was analysed by RT-PCR followed by Southern blotting with CYP 2C and GAPDH probes. Total cellular phenol-soluble proteins were analysed by western blotting with a polyclonal CYP 2C antibody. Autoradiograph is representative of three additional experiments; Ponceau S staining shows equal loading of each lane of the protein gel. **b**, Statistical summary (mean ± s.e.m., $n = 4$, $^*P < 0.05$) of the β-naphthoflavone-induced increase in CYP 2C. **c**, Amount of 11,12-EET determined by HPLC using endothelial cell extracts obtained following intraluminal incubation of coronary arteries with either solvent (48 h) or β-naphthoflavone (3 μM, 48 h). The results are presented as the median of duplicate samples; similar results were obtained in two additional experiments. **d**, PCA incubated with either solvent (48 h) or β-naphthoflavone (3 μM, 48 h) were cut into rings and preconstricted with U46619 (0.1–1 μM), and the EDHF-mediated relaxation to bradykinin was determined in the presence of diclofenac (10 μM) and N^{ω}nitro-L-arginine (300 μM). Results are presented as the mean ± s.e.m. of 4 separate experiments. **e**, PCA were cut into rings and preconstricted with U46619 (0.1–1 μM), and the EDHF-mediated relaxation to bradykinin was determined in the presence of diclofenac (10 μM) and N^{ω}nitro-L-arginine (300 μM), with or without sulphaphenazole (Sph; 10 μM). Results are presented as the mean ± s.e.m. of 6 separate experiments; $^{**}P < 0.01$ versus control.

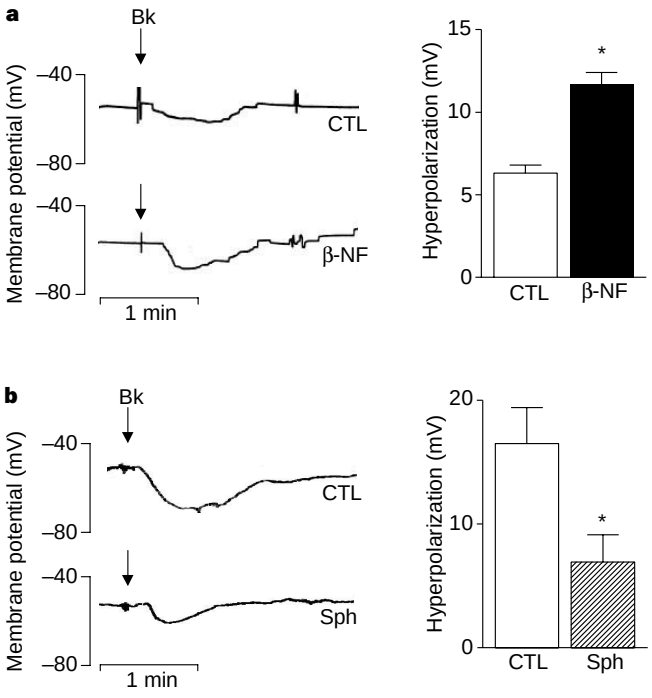

Figure 2 Increased expression of CYP 2C enhances, and sulphaphenazole inhibits, the EDHF-mediated hyperpolarization of PCA. The bradykinin (Bk; 100 nM)-induced hyperpolarization was determined in endothelium-intact PCA segments. Coronary artery segments were pretreated with **a**, solvent (CTL; 48 h) or β-naphthoflavone (β-NF; 3 μM, 48 h), or **b**, solvent or sulphaphenazole (Sph; 10 μM, 30 min). All experiments were performed in the presence of diclofenac (10 μM), N^{ω}nitro-L-arginine (300 μM) and U46619 (1 μM). Left, representative membrane-potential recordings; right, statistical summary of data obtained in 4–6 separate experiments; $^*P < 0.05$ versus control.

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muscle cells (50 ± 1.8 mV in control versus 51.5 ± 2.1 mV in β-naphthoflavone-treated PCA) or the hyperpolarization elicited by 11,12-EET (not shown). Sulphaphenazole, which selectively inhibits CYP 2C8/9 in human liver[17], significantly attenuated the bradykinin-induced EDHF-mediated relaxation of PCA rings (Fig. 1e) as well as EDHF-induced hyperpolarization (Fig. 2b), without affecting 11,12-EET-induced hyperpolarization (not shown).

CYP-derived metabolites of arachidonic acid synthesized in endothelial cells harvested from PCA were analysed by high-performance liquid chromatography (HPLC)[18]. Endothelial cells maintained under control conditions for 48 h generated 11,12-EET, and incubation with β-naphthoflavone induced a 3.8-fold increase in 11,12-EET generation (Fig. 1c). In some, but not all, β-naphthoflavone-treated endothelial cells 8,9-EET was also found, whereas 14,15- and 5,6-EET were not detected.

Although bradykinin-induced hyperpolarization of PCA was detectable for up to 60 h, the amplitude of the hyperpolarization decreased in a time-dependent manner upon incubation in culture medium, with hyperpolarizations of 17.3 ± 0.8, 8.6 ± 0.9 and 6.3 ± 0.5 mV being obtained after 6, 20 and 48 h, respectively ($P < 0.05$, $n = 8$). There was a corresponding shift to the right in the bradykinin-induced EDHF-mediated relaxation of PCA incubated for various times (compare Fig. 1e and 1d). In all experiments, the combination of charybdotoxin and apamin completely inhibited EDHF-induced hyperpolarization and relaxation (not shown). The combined application of barium and ouabain, which inhibits CYP-independent, EDHF-mediated hyperpolarization in the rat hepatic and mesenteric arteries[19], failed to affect bradykinin-induced, EDHF-mediated vascular responses (not shown).

Antisense oligonucleotides derived from the complementary DNA sequences of human CYP 2C8 and 2C9 (5′-GAGGAG-TGGGGCCAGGAGGGAG-3′) were used to modulate the expression of 2C protein in PCA. This DNA sequence is located 50 base pairs (bp) downstream of the translation-initiation site within the coding region and exhibits homology only to CYP sequences within the 2C family, including the porcine 2C34. Antisense (As) oligonucleotides markedly downregulated the expression of CYP 2C in PCA endothelial cells, whereas sense (Se) and scrambled (Scr) oligonucleotides slightly increased 2C expression (Fig. 3a). Identical results were obtained using cultured porcine aortic endothelial cells.

To determine whether the downregulation of CYP 2C8/34 selectively affected EDHF-mediated relaxation, experiments were performed using isometrically contracted PCA rings. Treatment of PCA with the transfection reagent alone or with sense or scrambled oligonucleotides did not affect either the combined EDHF/nitric oxide (NO) response (Fig. 3c) or responses mediated by NO (Fig. 3b) or EDHF alone (Fig. 3d). However, EDHF-mediated relaxation, assessed in the presence of both the NOS inhibitor N$^{\omega}$nitro-L-arginine and the COX inhibitor diclofenac, was selectively attenuated in rings from PCA treated with antisense oligonucleotides (Fig. 3c). Antisense oligonucleotide treatment enhanced the NO-mediated relaxation to bradykinin with or without sulphaphenazole (with diclofenac, $EC_{50} = -8.07 \pm 0.27$ M in control arteries and -8.91 ± 0.19 M in arteries treated with antisense oligonucleotides, $P < 0.01$, $n = 6$; with diclofenac and sulphaphenazole, $EC_{50} = -7.70 \pm 0.19$ M in control arteries and -9.43 ± 0.17 M in arteries treated with antisense oligonucleotides, $P < 0.001$,

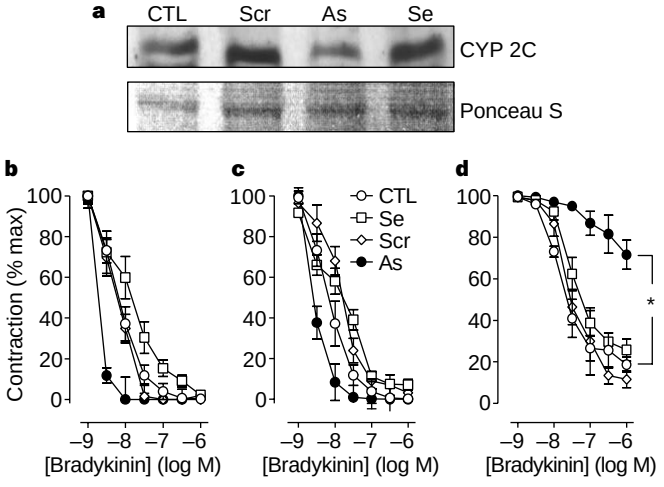

Figure 3 CYP 2C8/34 antisense oligonucleotides attenuate the expression of CYP 2C protein and the EDHF-mediated relaxation of PCA. **a**, Isolated PCA were intraluminally incubated with cationic liposomes in the absence (CTL) and presence of sense (Se), scrambled (Scr) or antisense (As) CYP 2C8/34 oligonucleotides for 3 h. Arteries were perfused for an additional 18–24 h with culture medium before isolation of endothelial cells and determination of CYP 2C expression by western blotting. Autoradiograph represents 4 additional experiments; Ponceau S staining shows equal loading of each lane. **b**, **c**, **d**, PCA treated with liposomes in the absence and presence of sense, scrambled and antisense CYP2C8 oligonucleotides were cut into rings and mounted in an organ chamber, and the relaxation to bradykinin was determined following contraction with U46619 (0.1–1 M). **b**, NO-mediated relaxation was assessed in the presence of diclofenac (10 μM) and sulphaphenazole (10 μM). **c**, NO- and EDHF-mediated relaxation was assessed in the presence of diclofenac (10 μM). **d**, EDHF-mediated relaxation was assessed in the presence of diclofenac and N$^{\omega}$nitro-L-arginine (300 μM). Results are presented as the mean ± s.e.m. of 6 separate experiments; *$P < 0.05$ versus control.

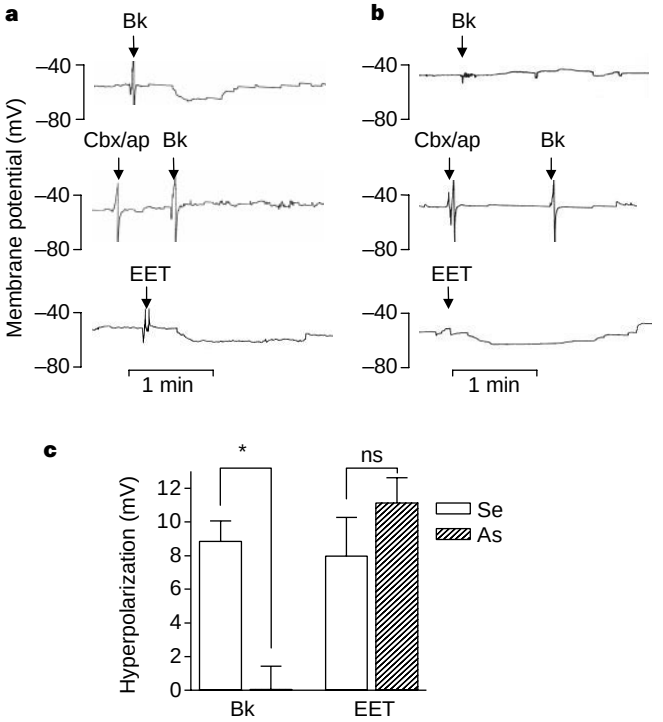

Figure 4 CYP 2C antisense oligonucleotides abolish bradykinin-induced, EDHF-mediated hyperpolarization of PCA smooth muscle cells. The effects of bradykinin (Bk; 100 nM), bradykinin with charybdotoxin (Cbx; 100 nM), apamin (ap; 100 nM) and 11,12-EET (EET; 1 μM) on the membrane potential of endothelium-intact porcine coronary artery segments were determined following treatment with CYP 2C sense oligonucleotides (**a**) and CYP 2C antisense oligonucleotides (**b**). **c**, Effects of sense (Se) and antisense (As) CYP 2C oligonucleotides on the hyperpolarization induced by bradkykinin and 11,12-EET. All experiments were performed in the presence of diclofenac (10 μM), N$^{\omega}$nitro-L-arginine (300 μM) and U46619 (1 μM). Results are presented as the mean ± s.e.m. of 4–8 separate experiments, *$P < 0.05$ versus sense.

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$n = 6$). Thus the downregulation and inhibition of CYP 2C8/34 potentiate NO-mediated relaxation, an effect that is probably due to decreased production of superoxide anions, which are a by-product of CYP activity[20,21].

Next we assessed the effect of CYP 2C8/34 downregulation on the bradykinin-induced hyperpolarization of PCA segments. In PCA treated with either the transfection reagent or sense oligonucleotides, bradykinin induced the hyperpolarization of smooth muscle cells (Fig. 4a). This hyperpolarization was dependent on the presence of the endothelium and was abolished by the inclusion of charybdotoxin and apamin in the perfusion solution (Fig. 4a). The CYP epoxygenase product, 11,12-EET, also elicited the hyperpolarization of PCA pretreated with sense oligonucleotides. In PCA pretreated with antisense oligonucleotides, bradykinin frequently (5 out of 8 PCA) elicited depolarization rather than hyperpolarization of vascular smooth muscle cells, whereas 11,12-EET induced hyperpolarization of similar magnitude to that observed in PCA treated with sense oligonucleotides (Fig. 4b).

Our data show that endothelial 11,12-EET production, as well as EDHF-mediated hyperpolarization and relaxation, in porcine coronary arteries can be selectively modulated by altering the expression and activity of CYP 2C8/34. This represents, to our knowledge, the first direct evidence for the existence of a CYP-dependent EDHF synthase in native endothelial cells of the coronary circulation. Moreover, as 11,12-EET was the main CYP 2C product generated by porcine coronary artery endothelial cells, this EET, which activates K^+_{Ca} channels and hyperpolarizes vascular smooth muscle cells, is a leading candidate for the 'coronary EDHF'. □

Methods

Cultured endothelial cells

Porcine aortic and human umbilical vein endothelial cells were isolated as described[6] and grown to confluence. Human lung microvascular and human coronary endothelial cells were purchased from Cell Systems/Clonetics. Confluent cells were then incubated with either solvent or β-naphthoflavone (3 μM, 48 h). Thereafter protein and/or total RNA were isolated.

Preparation of PCA

Porcine epicardial artery (PCA) segments (~40 mm length; mean external diameter 2.4–2.8 mm) were excised, side branches were sealed with surgical clips and the segment was cannulated at both ends and placed into vessel chambers. After equilibration, MEM containing 10% FCS, 2.5 μg ml^{-1} FITC-labelled antisense, sense or scrambled oligonucleotides and 20 μl ml^{-1} transfection reagent (Superfect, Qiagen) were applied luminally and left in contact for 3 h under a maintained transmural pressure of 90 mm Hg. Thereafter, PCA were perfused (5 ml h^{-1}) with MEM containing 2% FCS for 18–20 h. In a separate series of experiments, PCA were perfused with MEM containing β-naphthoflavone (3 μM, 48 h, 5 ml h^{-1}). All incubations were performed at 37 °C. Following incubation, half of each segment was cut into rings for organ chamber studies, precontracted with U46619 (0.1–1 μM), and the relaxation to bradykinin determined in the presence of diclofenac (10 μM) and with and without the NOS inhibitor, N$^{\omega}$nitro-L-arginine (300 μM) as described[22].

Isolation of RNA and protein from PCA

From the remaining PCA segment, we isolated endothelial cells or total RNA by intraluminal incubation with either dispase (2.4 U ml^{-1}, 45 min) or guanidine isothiocyanate. Random hexanucleotide primers were used for reverse transcription of equal amounts of RNA and the oligonucleotides used for PCR were derived from a porcine CYP 2C34 sequence (genebank accession no. U35843; upstream primer: AGACAACGAGCACCACTCTG; downstream primer: CTTGGGGATGAGGTAGTTT) which exhibited high homology to the human 2C8 sequence (genebank accession no. Y00498). PCR products were separated on a 1.5% TAE agarose gel and visualized by staining with ethidium bromide. To verify the DNA fragment, the PCR products were transferred to nylon membranes and hybridized with a ^{32}P-labelled PCR product generated using a plasmid containing the coding sequence of CYP 2C8 (provided by K. A. Pritchard Jr). Phenol-soluble protein prepared from isolated PCA endothelial cells was subjected to SDS–PAGE (8%) and western blotting using two different 2C epoxygenase (rat: CYP 2C11) polyclonal antibodies (provided by E. Morgan or purchased from Daiichi Pure Chemicals).

Analysis of CYP-derived products

Native PCA endothelial cells harvested using dispase, as above, were homogenized in 200 μl of 0.2 M KCl containing 0.01% butylated hydroxytoluene as an antioxidant and prostaglandin B_1 (PGB_1). CYP-derived eicosanoids were extracted three times by addition of an equal volume of diethyl ether containing 0.005% BHT and 0.1% acetic acid, and the combined organic phases were evaporated under a stream of nitrogen. Dried samples were dissolved in acetonitrile/methanol (80/20, v/v) and subjected to isocratic reversed-phase (RP) HPLC with online photodiode array detection (PDAD) as described[18]. RP-HPLC was carried out on octadecylsilyl columns (Hypersil, Shandon; 3-μm particles) with a mobile phase of acetonitrile/methanol/water/formic acid (54/8/38/0.001). CYP-derived metabolites were detected at 204 nm, identified by spectra plot on the peak maximum and co-elution with commercial standards, and assessed by an internal standard method using PGB_1 as standard. Analysis of full UV spectra (190–340 nm) provided by PDAD allowed accurate identification and quantification of eluting analytes by peak purity control and subtraction of possible co-eluting material.

Electrophysiological measurements

Following incubation with β-naphthoflavone or antisense, sense or scrambled oligonucleotides, PCA rings were slit and mounted in a heated chamber (37 °C) and maintained in HEPES-modified Tyrode solution containing diclofenac (10 μM) and N$^{\omega}$nitro-L-arginine (300 μM) and U46619 (1 μM) to mimic conditions in the organ chamber experiments. Smooth muscle membrane potential was recorded by impaling cells through the intima. Measurements of intracellular electrical potential were made with a high impedance amplifier and glass capillary microelectrodes (tip resistance 50–150 MΩ), filled with 3 M KCl. Criteria for a successful impalement included an abrupt drop in potential on impalement, followed by a stable negative potential for at least 1 min, and a rapid return to the previous potential upon withdrawal of the microelectrode.

Statistics

Data are expressed as mean ± s.e.m. Statistical evaluation was performed using Student's *t*-test for unpaired data, one-way analysis of variance (ANOVA) followed by a Bonferroni *t*-test, or ANOVA for repeated measures where appropriate. Values of $P < 0.05$ were considered statistically significant.

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Ascaris haemoglobin is a nitric oxide-activated 'deoxygenase'

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The parasitic nematode *Ascaris lumbricoides* infects one billion people worldwide. Its perienteric fluid contains an octameric haemoglobin[1–3] that binds oxygen nearly 25,000 times more tightly than does human haemoglobin[4,5]. Despite numerous investigations, the biological function of this molecule has remained elusive. The distal haem pocket contains a metal, oxygen and thiol[6], all of which are known to be reactive with nitric oxide. Here we show that *Ascaris* haemoglobin enzymatically consumes oxygen in a reaction driven by nitric oxide, thus keeping the perienteric fluid hypoxic. The mechanism of this reaction involves unprecedented chemistry of a haem group, a thiol and nitric oxide. We propose that *Ascaris* haemoglobin functions as a 'deoxygenase', using nitric oxide to detoxify oxygen. The structural and functional adaptations of *Ascaris* haemoglobin suggest that the molecular evolution of haemoglobin can be rationalized by its nitric oxide related functions.

Because *Ascaris* haemoglobin has such a high affinity for oxygen, it seems unlikely that its purpose is oxygen delivery. However, there is evidence that its function might involve nitric oxide (NO). First, large amounts of oxidized haemoglobin have been found in *Ascaris* perienteric fluid[4]. This is unexpected for an oxygen-avid haemoglobin[4]. However, NO oxidizes oxygen-ligated haem groups in haemoglobin[7], and NO synthase activity occurs in the hypodermis and muscle of *Ascaris*[8,9]; various nitrogen oxides (NO_x) are also found in the host gut[10]. Second, the distal E7 residue, which is histidine in haemoglobins from most higher organisms, is a glutamine in *Ascaris* haemoglobin. Substitution of the E7 histidine with glutamine increases ~1,000-fold the reactivity of oxidized myoglobin with NO[11]. Third, the crystal structure of an *Ascaris* haem domain[6] reveals a cysteine in close proximity to the ligand-binding site. NO is transferred between haem and thiol in human haemoglobin[7,12–14], and we find that fresh *Ascaris* perienteric fluid contains endogenous S-nitrosothiol (SNO). The presence of these components of NO biology indicate that NO may be involved in the function of *Ascaris* haemoglobin.

We constructed optical spectra for various liganded, unliganded and oxidized forms of *Ascaris* haemoglobin (AH), from which difference spectra were derived (not shown). Absorption-difference spectroscopy was used to examine the reaction of NO with the haemoglobin. NO was titrated against 6 μM haemoglobin (haem content) in 1.8-μM steps. Addition of NO resulted in the immediate oxidation of haemoglobin to methaemoglobin (Fig. 1a), whereas oxidation of haemoglobin by ferricyanide[4], which requires dissociation of liganded oxygen, requires several minutes. The peak ferric (Fe III) haem yield was seen after the addition of 19.8 μM NO (three NO molecules per haem) (Fig. 1a). Addition of further NO, to a total of 45 μM, induced the accumulation of AH (Fe III) NO. These data indicate that NO is directly oxidizing $AH(Fe^{II})O_2$ to methaemoglobin (reactions 1 and 2 below); that NO reacts with methaemoglobin to form $AH(Fe^{II})NO$ (reaction 3); and that additional reactions must be occurring.

$$AH(Fe^{II})O_2 \leftrightarrow AH(Fe^{III})O_2^- \quad (1)$$

$$AH(Fe^{III})O_2^- + NO^{\cdot} \leftrightarrow AH(Fe^{III}) + NO_3^- \quad (2)$$

$$AH(Fe^{III}) + NO^{\cdot} \leftrightarrow AH(Fe^{III})NO \quad (3)$$

We used photolysis-chemiluminescence to measure the NO content of 6 μM haemoglobin (haem content) following the stepwise addition of 45 μM NO, as described above. The haemoglobin contained 7.8 μM NO, of which 5.1 μM (65%) was SNO (results not shown), consistent with an equilibrium that is known to occur between Fe(III)NO in mammalian haemoglobin and SNO (reaction 4). However, the transfer of NO^+ from haem to thiol would inevitably be coupled to the binding of oxygen (reaction 5), which was not detected. Moreover, most of the added NO did not form either a chemiluminescence-detectable or spectrally active nitrosyl species.

$$AH(Fe^{III})NO + (Cys)S^- \leftrightarrow AH(Fe^{II}) + (Cys)SNO \quad (4)$$

$$AH(Fe^{II}) + O_2 \leftrightarrow AH(Fe^{II})O_2 \quad (5)$$

Accordingly, we hypothesized that NO might be consumed in a reaction with oxygen to form nitrate (reaction 6), but this reaction would require a source of electrons (reaction 7).

$$AH(Fe^{II})O_2 + (Cys)SNO \leftrightarrow AH(Fe^{III}) + (Cys)S^{\cdot} + NO_3^- \quad (6)$$

$$AH(Fe^{III}) + (Cys)S^{\cdot} + e^- \leftrightarrow AH(Fe^{II}) + (Cys)S^- \quad (7)$$

Previous work has shown that *Ascaris* haemoglobin can reduce oxidized cytochrome *c* in an NADPH-dependent manner[15]. NADPH was found to increase the efficiency with which haemoglobin was oxidized by NO. Moreover, very little $AH(Fe^{III})NO$

Table 1 Endproduct analysis of NO metabolism by *Ascaris* haemoglobin

Concentration of DEANO added (μM)	NO_2^- (μM), no haemoglobin	NO_3^- (μM), no haemoglobin	NO_2^- (μM), with haemoglobin	NO_3^- (μM) with haemoglobin
~2	0.0 ± 0.09	0.0 ± 0.7	0.0 ± 0.27	1.2 ± 0.11
~4	0.8 ± 0.04	1.0 ± 0.12	0.1 ± 0.17	8.5 ± 0.12
~8	5.7 ± 0.52	8.1 ± 0.27	2.4 ± 0.07	11.6 ± 0.29

Varying amounts of DEANO were added to solutions containing 500 μM NADPH with or without *Ascaris* haemoglobin (1.5 μM haem content). Yields of nitrite and nitrate were determined by the Greiss reaction and high-performance capillary electrophoresis. Values are mean ± standard error for three experiments.

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