Depletion of iNOS-derived nitric oxide by prostaglandin H synthase-2 in inflammation-activated J774.2 macrophages through lipohydroperoxidase turnover

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PGHS-2 (prostaglandin H synthase-2) is induced in mammalian cells by pro-inflammatory cytokines in tandem with iNOS [high-output ('inducible') nitric oxide synthase], and is co-localized with iNOS and nitrotyrosine in human atheroma macrophages. Herein, murine J774.2 macrophages incubated with lipopolysaccharide and interferon γ showed induction of PGHS-2 and generated NO using iNOS that could be completely depleted by 12(S)-HPETE [12(S)-hydroperoxyeicosatetraenoic acid; 2.4 μ M] or hydrogen peroxide (500 μ M) (0.42 ± 0.084 and 0.38 ± 0.02 nmol $\cdot$ min⁻¹ $\cdot$ 10⁶ cells⁻¹ for HPETE and H₂O₂ respectively). COS-7 cells transiently transfected with human PGHS-2 also showed HPETE- or H₂O₂-dependent NO decay (0.44 ± 0.016 and 0.20 ± 0.04 nmol $\cdot$ min⁻¹ $\cdot$ 10⁶ cells⁻¹ for 2.4 μ M HPETE and 500 μ M H₂O₂ respectively). Finally, purified PGHS-2 consumed NO in the presence of HPETE or H₂O₂ (168 and 140 μ M $\cdot$ min⁻¹ $\cdot$ μ M enzyme⁻¹ for HPETE and H₂O₂ respectively), in a haem-dependent manner, with 20 nM enzyme consuming up to 4 μ M NO. K_m (app) values for NO and 15(S)-HPETE were 1.7 ± 0.2 and 0.45 ± 0.16 μ M respectively. These data indicate that PGHS-2 catalytically consumes NO during peroxidase turnover and that pro-inflammatory cytokines simultaneously up-regulate NO synthesis and degradation pathways in murine macrophages. Catalytic NO consumption by PGHS-2 represents a novel interaction between NO and PGHS-2 that may impact on the biological effects of NO in vascular signalling and inflammation.

Key words: cyclo-oxygenase, inflammation, nitric oxide, peroxidase, prostaglandin H synthase-2 (PGHS-2), reactive nitrogen species.

INTRODUCTION

Nitric oxide plays essential roles in vascular homeostasis through maintaining vascular tone and inhibiting leucocyte and platelet activation. While pathways of NO generation are well known, mechanisms of NO removal in specific cell types are only now being elucidated. We found previously that human PGHS (prostaglandin H synthase)-1 can consume NO via peroxidase turnover and that this attenuates its inhibitory effects on platelet function [1]. In contrast with PGHS-1, PGHS-2 is induced at high levels by pro-inflammatory cytokines in tandem with the high-output NOS (NO synthase) isoform, iNOS ('inducible' NOS). Indeed, in human atherosclerotic lesions PGHS-2 co-localizes with iNOS and nitrotyrosine, particularly in macrophages [2]. This makes it especially interesting as a candidate for controlling NO bioactivity, and several observations suggest that PGHS-2-dependent NO consumption may occur in cells and *in vivo*. Inhibition of PGHS-2 elevates iNOS-derived NO and promotes its toxicity in LPS (lipopolysaccharide)-dependent meningitis or LPS/IFN γ (interferon γ)-treated RAW 264.7 macrophages [3,4]. Also, administration of PGHS-2-selective inhibitors acutely increases NO bioactivity in hypertension or coronary artery disease, where PGHS-2 elevation is a consistent finding [5–9]. However, the mechanisms underlying these observations have not been characterized. This prompted us to examine the ability of both purified and cellular PGHS-2

to catalytically consume NO during turnover, particularly when generated intracellularly by iNOS in macrophages.

Herein, murine PGHS-2 and cells transfected with hPGHS-2 (human PGHS-2) consumed exogenously added NO via peroxidase turnover. Also, LPS/IFN γ -treated murine J774.2 macrophages rapidly consumed NO in the presence of HPETE (hydroperoxyeicosatetraenoic acid), H₂O₂ or arachidonate. The capacity of inflammation-activated J774.2 macrophages to consume NO far exceeded the synthetic capacity of iNOS. These data reveal a functional interplay between PGHS-2 and NO that leads to complete removal of endogenously synthesized NO in murine macrophages, and provide a potential mechanistic explanation for observations of improved NO bioactivity following administration of PGHS-2 inhibitors in inflammation and vascular disease.

EXPERIMENTAL

Materials

Recombinant murine PGHS-2 was purified as described in [10]. The apoenzyme was reconstituted with haematin (haematin/apoenzyme, 2:1) in 80 mM Tris, pH 7.8, for 20 min at 22°C. 15(S)-HPETE, 12(S)-HPETE and 12(S)-HETE [12(S)-hydroxy-eicosatetraenoic acid] were from Cayman Chemical (Ann Arbor,

Abbreviations used: HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; IFN γ , interferon γ ; iNOS, high-output ('inducible') nitric oxide synthase; LPS, lipopolysaccharide; L-NAME, N^G-nitro-L-arginine methyl ester; NOS, nitric oxide synthase; PGG₂, prostaglandin G₂; PGHS, prostaglandin H synthase; hPGHS-2, human PGHS-2.

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MI, U.S.A.). For enzyme assays, the sodium salt of HPETE was prepared in 10 % (v/v) methanol/0.01 M NaOH.

Generation of hPGHS2pcDNA3 construct

HeLa OHIO cells were treated with 10 nM phorbol for 4 h to induce expression of hPGHS-2 [11]. Total RNA was extracted and purified using the UltraspecTM RNA isolation system (Biotecx, Houston, TX, U.S.A.). First-strand cDNA synthesis was carried out using ImProm-IITM reverse transcriptase (Promega, Southampton, U.K.). The PGHS-2 coding region and flanking sequence were amplified by PCR using Elongase[®] (Invitrogen, Paisley, Scotland, U.K.) and the following sense and antisense primers, which contain restriction sites for *Bam*HI and *Eco*RI respectively (in italics): sense, *GGATCCAGACGCCCTCAGACAGCAAAGCCT*; antisense, *GAATTCCTCCGCAACAGGAGTACTGACTTCTG*.

Amplified hPGHS-2 cDNA was desalted using the QIAquick PCR purification system (Qiagen, Crawley, Sussex, U.K.) and cloned into pGEM[®]-T (Promega). Positive clones were confirmed by sequencing using Big Dye chemistry (Applied Biosystems). The hPGHS-2 insert was excised by digestion with *Eco*RI and *Bam*HI (Promega), purified by gel extraction and subcloned into the mammalian expression vector pcDNA3 (Promega). Recombinant pcDNA3 without the insert was also prepared for control transfections.

Expression of PGHS-2 in COS-7 cells

COS-7 cells were transiently transfected at 70 % confluence with hPGHS2pcDNA3, using the ProFectin[®] Mammalian Transfection System Calcium Phosphate (Promega). DNA (17 µg) in 125 mM CaCl₂/Hepes-buffered saline was added dropwise to cells, then replaced by fresh medium after 5 h, and cells were harvested at 70 h and washed three times with PBS, pH 7.4.

Induction of PGHS-2 and iNOS in J774.2 cells

Cells were incubated with 1 ng · ml⁻¹ IFN γ and 10 ng · ml⁻¹ LPS for 16 h before being harvested and washed four times with PBS. Western blotting (5–10 ng of protein) was performed using goat anti-hPGHS-2 polyclonal antibody (1:500; Santa Cruz Biotech) or rabbit anti-NOS2 polyclonal antibody (1:500; Santa Cruz Biotech) and visualized using ECL[®] (Amersham), following incubation with anti-goat or anti-rabbit IgG respectively conjugated to horseradish peroxidase [12].

Preparation and analysis of NO

Anaerobic solutions of NO were prepared by bubbling NO gas through argon-saturated deionized water. Contaminating NO₂ and N₂O₃ were removed by flowing through 1 M NaOH. NO concentrations were measured electrochemically using an NO sensor (Harvard AmiNO100 or AmiNO700 with inNO meter; Harvard Apparatus, Edenbridge, Kent, U.K.). Where NO loss was not linear, rates are given as first-order rate constants (k_{obs}). For all k_{obs} values, the square of the Pearson product moment correlation coefficient (r) of the slope of the replotted data was greater than 0.9, confirming that the reaction was first order. Where NO consumption was linear, NO disappearance was determined and the background rate of NO loss subtracted. For measurement of NO consumption by purified PGHS-2, NO was added to 0.5 ml of 80 mM Tris, pH 7.8, at 37 °C with or without 15(S)-HPETE or H₂O₂ with stirring. Once the NO response had stabilized, haematin, apo-PGHS-2 or holo-PGHS-2 was added and NO decay

rates were monitored. NO consumption by cells was measured following addition of H₂O₂ or HPETE to PBS containing (1–5) × 10⁶ cells · ml⁻¹, 1 mM L-NAME (*N*^G-nitro-L-arginine methyl ester), 1 mM CaCl₂ and 0.95 µM NO at 37 °C with stirring. NO production by J774.2 cells was measured in the absence of L-NAME following addition of 1 mM L-arginine to PBS containing 1 mM CaCl₂ and 10⁷ cells · ml⁻¹ at 37 °C with stirring.

Lipid extractions, absorbance spectroscopy of 15(S)-HPETE and calculation of Michaelis–Menten parameters

For analysis of conjugated dienes following PGHS-2 peroxidase turnover, 15(S)-HPETE (5 µM) was incubated in Tris, pH 7.8, for 5 min with or without the addition of 9.6 nM PGHS-2 and 7.6 µM NO, in triplicate. Following a 5 min incubation, samples were acidified and extracted twice using 2 vol. of diethyl ether. After drying under N₂, samples were solubilized in methanol and scanned from 220 to 350 nm, using methanol as reference. Michaelis–Menten parameters were calculated using Enzfitter (Elsevier-Biosoft, Cambridge, U.K.), following determination of initial rates of NO consumption at various NO or 15(S)-HPETE concentrations, using 9.6 nM PGHS-2.

RESULTS

Characterization of NO loss in the electrode system

NO decay in aerobic buffer at 37 °C followed first-order kinetics, with k_{obs} values of $(1.81 \pm 0.28) \times 10^{-3} \text{ s}^{-1}$ and $(7.86 \pm 1.0) \times 10^{-3} \text{ s}^{-1}$ ($r^2 > 0.9$) for the AmiNO100 and AmiNO700 probes respectively ($n = 3$, means $\pm$ S.D.). This indicates that NO oxidation by the electrode and diffusion into the gas phase are the main causes of NO decay in these experiments. Using these k_{obs} values, rates of background NO loss could be calculated and subtracted from all experimental values.

LPS/IFN γ treatment of murine monocytes stimulates lipohydroperoxidase-dependent NO consumption

J774.2 cells were incubated with IFN γ /LPS for 16 h to induce PGHS-2, as described previously (Figure 1A, left panel) [13]. In experiments measuring exogenous NO decay for cytokine-treated cells, 1 mM L-NAME was routinely added to inhibit endogenous iNOS activity. Rates of NO decay in the presence of J774.2 cells (10⁶ cells · ml⁻¹) were increased over background [buffer alone, $k_{\text{obs}} = (1.5 \pm 0.1) \times 10^{-3} \text{ s}^{-1}$], but were not significantly different for LPS/IFN γ -treated and control cells, and were still first order [$k_{\text{obs}} = (6.1 \pm 1.7) \times 10^{-3} \text{ s}^{-1}$ and $(6.7 \pm 0.6) \times 10^{-3} \text{ s}^{-1}$ for treated and control cells respectively]. At 0.6 µM NO, this gave NO consumption rates of 0.16 ± 0.06 and $0.19 \pm 0.02 \text{ nmol} \cdot \text{min}^{-1} \cdot 10^6 \text{ cells}^{-1}$ for LPS/IFN γ -treated and control cells respectively, indicating a low-level endogenous NO scavenging capacity of the cells that is unchanged by cytokine treatment. Addition of 2.4 µM 15(S)-HPETE to control cells did not increase NO decay. However, addition of 15(S)-HPETE to LPS/IFN γ -treated J774.2 cells immediately caused a significant increase in cell-dependent NO consumption ($0.42 \pm 0.084 \text{ nmol} \cdot \text{min}^{-1} \cdot 10^6 \text{ cells}^{-1}$ after subtraction of cell-dependent rates) (Figures 1B and 1C). Using 500 µM H₂O₂ as substrate instead of 15(S)-HPETE, increased rates of NO decay were found for both cell populations; however, these were significantly higher for cytokine-induced cells (0.23 ± 0.06 and $0.38 \pm 0.02 \text{ nmol} \cdot \text{min}^{-1} \cdot 10^6 \text{ cells}^{-1}$ for control and LPS/IFN γ -treated cells respectively, after subtraction of cell-dependent rates) (Figures 2A and 2B). Addition of H₂O₂ alone in the absence of cells did not accelerate NO decay (results not shown).

Figure 1 Cytokine induction of PGHS-2 in macrophages induces lipohydroperoxidase-dependent NO consumption

(A) PGHS-2 or iNOS expression was examined by Western blotting following treatment of J774.2 macrophages with $1 \text{ ng} \cdot \text{ml}^{-1}$ IFN γ and $10 \text{ ng} \cdot \text{ml}^{-1}$ LPS for 16 h. T, cytokine-treated cells; C, control cells. Molecular-mass sizes are indicated in kDa. (B) 15(S)-HPETE ($2.4 \mu\text{M}$) was added to 1×10^6 J774.2 cells in the presence of 1 mM L-NAME, 1 mM CaCl $_2$ and $0.8 \mu\text{M}$ NO in 1 ml of PBS ($n = 3$; means $\pm$ S.D.). Bars represent rates of NO decay for cells in the presence or absence of HPETE, as appropriate. *** $P < 0.001$ compared with control (unpaired t test). (C) Representative traces from (B): (i) NO decay in 10^6 cytokine-treated J774 cells $\cdot \text{ml}^{-1}$, 1 mM L-NAME and 1 mM CaCl $_2$ in PBS; (ii) $2.4 \mu\text{M}$ 15(S)-HPETE was added to cytokine-treated cells in (i); (iii) NO decay in 10^6 control J774 cells $\cdot \text{ml}^{-1}$, 1 mM L-NAME and 1 mM CaCl $_2$ in PBS; (iv) $2.4 \mu\text{M}$ 15(S)-HPETE was added to control cells in (iii).

Peroxidase turnover depletes endogenously synthesized NO in LPS/IFN γ -treated murine monocytes

Cytokine treatment of J774 cells causes iNOS to be induced in tandem with PGHS-2 (Figure 1A, right panel). In the absence of L-NAME, and with 1 mM L-arginine added as an iNOS substrate, LPS/IFN γ -treated J774.2 cells, but not untreated controls, produced NO, which reached a plateau of approx $0.3 \mu\text{M}$ after a few minutes and decreased slowly over several minutes thereafter (Figure 2C, and results not shown). Following addition of $2.4 \mu\text{M}$ 15(S)-HPETE, all endogenously synthesized NO was rapidly and totally consumed (Figure 2C). As controls, the NO was also effectively removed on addition of $2.5 \mu\text{M}$ oxyHb or 1 mM L-NAME (Figure 2C).

PGHS-2-expressing COS-7 cells consume NO

COS-7 cells transiently transfected with hPGHS-2 contained immunoreactive enzyme that was absent from cells transfected with empty vector (Figure 3A). NO disappearance in the presence of 3×10^6 COS-7 cells $\cdot \text{ml}^{-1}$ (either PGHS-2- or neo-transfected) was increased slightly over background (results not shown). However, rates were not significantly different for PGHS-2- and neo-transfected cells, indicating that this was a property

Figure 2 H $_2$ O $_2$ induces NO consumption, and lipohydroperoxidase turnover depletes endogenously generated NO, in cytokine-treated macrophages

(A) H $_2$ O $_2$ ($500 \mu\text{M}$) was added to 2×10^6 cells, 1 mM L-NAME and 1 mM CaCl $_2$ in 1 ml of PBS ($n \geq 3$; means $\pm$ S.D.). Bars represent rates of NO decay for cells in the presence or absence of H $_2$ O $_2$, as appropriate. *** $P < 0.05$ compared with control (unpaired t test). (B) Representative traces from (A): (i) NO decay in PBS containing 2×10^6 cytokine-treated J774 cells $\cdot \text{ml}^{-1}$, 1 mM L-NAME and 1 mM CaCl $_2$; (ii) $500 \mu\text{M}$ H $_2$ O $_2$ was added to cytokine-treated cells in (i); (iii) NO decay in 2×10^6 control J774 cells $\cdot \text{ml}^{-1}$, 1 mM L-NAME and 1 mM CaCl $_2$; (iv) $500 \mu\text{M}$ H $_2$ O $_2$ was added to control cells in (iii). (C) OxyHb ($3 \mu\text{M}$) (i), $2.4 \mu\text{M}$ 15(S)-HPETE (ii) or 1 mM L-NAME (iii) was added to 10^7 cytokine-treated J774 cells $\cdot \text{ml}^{-1}$, 1 mM L-arginine and 1 mM CaCl $_2$ in PBS.

intrinsic to the cell line itself (results not shown). Addition of $2.4 \mu\text{M}$ 15(S)-HPETE caused a small increase in NO consumption by control cells ($0.20 \pm 0.079 \text{ nmol} \cdot \text{min}^{-1} \cdot 10^6 \text{ cells}^{-1}$ after subtraction of cell-dependent rates) (Figures 3B and 3C). In contrast, 15(S)-HPETE stimulated significantly higher rates of NO decay when added to hPGHS2pcDNA3-transfected cells ($0.44 \pm 0.016 \text{ nmol} \cdot \text{min}^{-1} \cdot 10^6 \text{ cells}^{-1}$ after subtraction of cell-dependent rates) (Figures 3B and 3C). Similarly, $500 \mu\text{M}$ H $_2$ O $_2$ increased NO decay to a significantly greater extent in hPGHS2pcDNA3-transfected COS-7 cells than in neo-transfected controls (0.20 ± 0.04 and $0.10 \pm 0.03 \text{ nmol} \cdot \text{min}^{-1} \cdot 10^6 \text{ cells}^{-1}$ respectively, after subtraction of cell-dependent rates) (Figures 4A and 4B).

Purified recombinant PGHS-2 consumes NO during peroxidase turnover

In the absence of peroxide substrate, haem-reconstituted PGHS-2 did not accelerate NO decay (Figure 5, Table 1). However,

Figure 3 COS-7 cells transfected with hPGHS-2 consume NO via lipohydroperoxidase turnover

(A) Western blot of COS-7 cells transfected with hPGHS-2 (T) or neo-transfected control cells (C). (B) 15(S)-HPETE (2.4 μ M) was added to 1 ml of PBS containing 3×10^6 cells, 1 mM CaCl₂ and 0.8 μ M NO ($n \geq 3$; means $\pm$ S.D.). Bars represent rates of NO decay for cells in the presence or absence of HPETE, as appropriate. *** $P < 0.01$ compared with control (unpaired t test). (C) Representative traces from (B): (i) NO decay in PBS containing 3×10^6 PGHS-2-transfected COS-7 cells and 1 mM CaCl₂; (ii) 2.4 μ M 15(S)-HPETE was added to PGHS-2-transfected COS-7 cells in (i); (iii) NO decay in PBS containing 3×10^6 control COS-7 cells and 1 mM CaCl₂; (iv) 2.4 μ M 15(S)-HPETE was added to control COS-7 cells in (iii).

either 15(S)-HPETE or H₂O₂ stimulated PGHS-2-dependent NO disappearance significantly (168 and 140 μ M $\cdot$ min⁻¹ $\cdot$ μ M⁻¹ PGHS-2 for 6.2 μ M HPETE and 500 μ M H₂O₂ respectively, after subtraction of background). Since amounts of PGHS-2 (up to 20 nM) added to the assay were far below amounts of NO consumed (up to 4 μ M), peroxide-dependent NO consumption was a catalytic process. Hydroperoxide-stimulated NO consumption required haem reconstitution, indicating that NO was acting as a substrate for PGHS-2 peroxidase turnover (Figure 5, Table 1). Plotting initial rates of NO consumption at various substrate concentrations in the presence of 9.6 nM PGHS-2 gave K_m (app) values for NO and 15(S)-HPETE of 1.7 ± 0.2 μ M and 0.45 ± 0.16 μ M respectively (Figures 6A and 6B). Finally, spectral analysis of 15(S)-HPETE before and after PGHS-2-dependent NO consumption revealed no loss of conjugated diene spectrum, with the absorbance maximum at 235 nm being retained (Figure 6C). This is consistent with NO acting as a reducing peroxidase substrate, stimulating reduction of HPETE to HETE.

DISCUSSION

The present study shows that PGHS-2 peroxidase turnover can completely deplete endogenously synthesized NO in cytokine-activated J774.2 macrophages (Figures 1 and 2). Catalytic NO consumption represents a novel activity of PGHS-2 and provides

Figure 4 COS-7 cells transfected with hPGHS-2 consume NO following H₂O₂ addition faster than controls

(A) NO consumption of COS-7 cells was determined as described in the Experimental section, following addition of 500 μ M H₂O₂ to 1 ml of PBS containing 5×10^6 cells and 1 mM CaCl₂ ($n \geq 3$; means $\pm$ S.D.). Bars represent rates of NO decay for cells in the presence or absence of H₂O₂, as appropriate. *** $P < 0.05$ compared with control (unpaired t test). (B) Representative traces from (A): (i) NO decay in PBS containing 5×10^6 PGHS-2-transfected COS-7 cells and 1 mM CaCl₂; (ii) 500 μ M H₂O₂ was added to PGHS-2-transfected COS-7 cells in (i); (iii) NO decay in PBS containing 5×10^6 control COS-7 cells and 1 mM CaCl₂; (iv) 500 μ M H₂O₂ was added to control COS-7 cells in (iii).

a mechanistic explanation for previous observations of improved NO bioactivity following PGHS-2 inhibition *in vivo* and *in vitro* [3,4,8,9].

PGHS isoforms exhibit two catalytic activities, a cyclo-oxygenase activity that converts arachidonate into PGG₂ (prostaglandin G₂), and a haem-dependent peroxidase activity that reduces PGG₂ to PGH₂. In addition to PGG₂, other hydroperoxides can serve as substrates, including lipid hydroperoxides (HPETEs), organic hydroperoxides and, to a lesser extent, H₂O₂. We showed previously that PGHS-1 could consume NO [1]. However, while PGHS-1 and PGHS-2 share many similarities, they are also both structurally and catalytically quite different. For example, they share only 60% identity at the protein level, and catalytic differences include: (i) differences in the threshold for hydroperoxidase activation of cyclo-oxygenase activity (10-fold lower for PGHS-2); (ii) negative allosteric regulation at low concentrations of arachidonic acid for PGHS-1 cyclo-oxygenase (at 500–1000 nM); (iii) depending on substrate concentration, PGHS-2 peroxidase catalyses different ratios of one- and two-electron reduction of HPETE; and (iv) PGHS-2 can oxidize additional substrates, such as arachidonyl ethanolamide, whereas PGHS-1 cannot [14–17]. Based on this, differences in kinetic parameters for the interaction of NO with PGHS-2 compared with PGHS-1 are expected. Finally, at a biological level, PGHS-2 is expressed in a very different manner (i.e. in response to pro-inflammatory

Figure 5 Purified murine PGHS-2 catalytically consumes NO via peroxidase turnover

(A) (i) Decay of $3.8 \mu\text{M}$ NO in Tris buffer, pH 7.8, at 37°C ; (ii) decay of NO in buffer with $6.2 \mu\text{M}$ 15(S)-HPETE; (iii) purified PGHS-2 (20 nM) was added to buffer containing $3.8 \mu\text{M}$ NO and $6.2 \mu\text{M}$ 15(S)-HPETE as shown. (B) (i) Purified murine PGHS-2 (20 nM) was added to buffer with $3.8 \mu\text{M}$ NO alone; (ii) apo-PGHS-2 (20 nM) was added to buffer containing $6.2 \mu\text{M}$ 15(S)-HPETE and $3.8 \mu\text{M}$ NO; (iii) haematin (40 nM) was added to buffer containing $6.2 \mu\text{M}$ 15(S)-HPETE and $3.8 \mu\text{M}$ NO. (C) (i) Decay of NO in buffer containing $500 \mu\text{M}$ H_2O_2 ; (ii) purified PGHS-2 (20 nM) was added to buffer containing $500 \mu\text{M}$ H_2O_2 and $3.8 \mu\text{M}$ NO; (iii) apo-PGHS-2 (20 nM) was added to buffer containing $500 \mu\text{M}$ H_2O_2 and $3.8 \mu\text{M}$ NO; (iv) haematin (40 nM) was added to buffer containing $500 \mu\text{M}$ H_2O_2 and $3.8 \mu\text{M}$ NO.

cytokines) and in different cell types. Therefore the biological consequences of PGHS-2-dependent NO consumption will be very different from those of PGHS-1-dependent NO consumption. For these reasons, it is important to determine whether PGHS-2, independently of PGHS-1, can consume NO, and the potential impact of this on resulting cellular NO levels.

In the present study, recombinant murine PGHS-2 catalytically consumed NO in a haem-dependent manner that required

Table 1 NO uptake by purified PGHS-2

NO ($3.8 \mu\text{M}$) was added to 80 mM Tris buffer, pH 7.8, at 37°C containing H_2O_2 ($500 \mu\text{M}$) or 15(S)-HPETE ($6.2 \mu\text{M}$). Once the electrode response had stabilized, PGHS-2 (20 nM), apo-PGHS-2 (20 nM) or haematin (40 nM) was added, and rates of NO consumption were recorded. *For these samples, rates of NO loss at $3.0 \mu\text{M}$ were determined using the calculated first-order rate constant (k_{obs}). For all other samples, initial linear rates of NO loss on addition of PGHS-2 were determined. Values are means $\pm$ S.D.; $n = 3$. For all k_{obs} values, $r^2 = 0.99$. ND, not determined.

Sample	Rate ($\text{nmol} \cdot \text{min}^{-1}$)	k_{obs} (s^{-1})
Background*	0.7 ± 0.09	$(7.86 \pm 1.0) \times 10^{-3}$
+ HPETE*	0.6 ± 0.03	$(6.93 \pm 0.36) \times 10^{-3}$
+ H_2O_2 *	0.77 ± 0.1	$(8.6 \pm 1.2) \times 10^{-3}$
+ PGHS-2*	0.75 ± 0.02	$(8.5 \pm 0.2) \times 10^{-3}$
+ Apo-PGHS-2 + HPETE*	0.58 ± 0.05	$(6.54 \pm 0.6) \times 10^{-3}$
+ Haematin + HPETE	0.8 ± 0.15	ND
+ PGHS-2 + HPETE	2.38 ± 0.28	ND
+ Apo-PGHS-2 + H_2O_2 *	0.81 ± 0.07	$(9.06 \pm 0.8) \times 10^{-3}$
+ Haematin + H_2O_2 *	0.8 ± 0.01	$(8.96 \pm 1.3) \times 10^{-3}$
+ PGHS-2 + H_2O_2	2.1 ± 0.3	ND

hydroperoxide substrates, i.e. HPETE or H_2O_2 (Figure 5, Table 1). This confirms that NO can act as a reducing substrate for PGHS-2 peroxidase activity [1,18]. Far lower concentrations of HPETE than of H_2O_2 were required, consistent with previous reports of K_m values for H_2O_2 being 50–100 times greater than for HPETEs [19–21]. Therefore HPETE will be the favoured substrate for PGHS-2-dependent NO consumption in cells. Kinetic analysis of NO consumption at various HPETE concentrations revealed a K_m (app) for 15(S)-HPETE of $0.45 \pm 0.16 \mu\text{M}$, which is almost 10-fold lower than that calculated for the hydroperoxidase-dependent consumption of NO by ovine PGHS-1 of $3.3 \pm 0.3 \mu\text{M}$ (Figure 6) [1]. Previous K_m values for the reduction of lipid hydroperoxides by PGHS-1 are in the range $5\text{--}20 \mu\text{M}$, similar to our calculated value for hydroperoxide-dependent NO consumption by PGHS-1 [19–21]. Although we were unable to find calculated K_m values for lipid hydroperoxide reduction by PGHS-2, hydroperoxide initiator requirements for cyclo-oxygenase turnover, which are far below than those required for full peroxidase activity, are approx. 10-fold lower for PGHS-2 than for PGHS-1 (e.g. 2 nM and 20 nM respectively) [14]. This suggests that PGHS-2 will preferentially consume NO over PGHS-1 under conditions of low intracellular peroxide levels. Indeed, previous studies have shown that, in cells where HPETE is metabolized rapidly by glutathione peroxidase, PGHS-2 functions far more effectively than PGHS-1, due to its higher affinity for HPETEs. This enables PGHS-2 to compete effectively with glutathione peroxidases for HPETE reduction and function under conditions where PGHS-1 is catalytically inactive (Figure 6) [1,18,22,23].

On varying the NO concentration, a K_m (app) for NO of $1.7 \pm 0.28 \mu\text{M}$ was determined (Figure 6). This is similar to the K_m (app) for NO calculated for ovine PGHS-1 of $2.2 \pm 0.9 \mu\text{M}$ (V. B. O'Donnell, unpublished work). These concentrations of NO are expected to occur *in vivo*, particularly under inflammatory conditions following induction of iNOS [24]. Finally, following peroxidase-dependent NO decay, the conjugated diene spectrum of HPETE was retained (Figure 6C) [1]. While PGHS-1 catalyses predominantly the two-electron reduction of lipid hydroperoxides, PGHS-2 can catalyse either one- or two-electron reduction, with the proportion of one-electron reduction products increasing at higher HPETE concentrations. Thus at $2.5 \mu\text{M}$ HPETE the enzyme catalyses $>97\%$ two-electron reduction, with 60% reduction at $50 \mu\text{M}$ HPETE [15]. Our experiments,

Figure 6 Calculation of kinetic parameters for HPETE-dependent NO consumption by PGHS-2

(A) K_m (app) was determined by measuring initial rates of NO consumption by 9.6 nM PGHS-2 at various 15(S)-HPETE concentrations. Michaelis-Menten parameters were calculated using Enzfitter (Elsevier-Biosoft). Rates of NO consumption are plotted as v against $v/[S]$. (B) K_m (app) was determined as described in (A) but by varying the NO concentration. (C) HPETE (5 μ M) was incubated for 5 min with or without 9.6 nM PGHS-2 and 7.2 μ M NO in Tris buffer, pH 7.8, before being extracted using acidified diethyl ether, as described in the Experimental section, and reconstituted in methanol. Samples were scanned from 200 to 350 nm using methanol as reference: (a) control, (b) with PGHS-2 plus NO.

conducted at 5 μ M HPETE, showed complete retention of the conjugated diene spectrum, consistent with reduction of HPETE to HETE, as found previously for PGHS-1 [1].

IFN γ /LPS-treated murine macrophages that express PGHS-2, but not untreated controls, consumed NO following addition of HPETE (Figure 1). Untreated control cells did not express detectable PGHS-1 or -2 (Figure 1 and results not shown). Using high concentrations of H₂O₂ as substrate, NO consumption was also observed; however, rates were lower and some H₂O₂-dependent NO consumption by control cells was also found (Figure 2). This may indicate activity of additional peroxidases that can reduce H₂O₂ but not HPETE, e.g. myeloperoxidase [25,26]. However, while previous studies showed that purified myeloperoxidase can consume NO, it has not been found to consume NO in intact cells, for example neutrophils [27], and it is not known to be expressed in J774 cells. Arachidonate

also stimulated NO decay by LPS/IFN γ -treated cells which could be partially inhibited by aspirin or celecoxib (results not shown); however, rates were variable, and it was not possible to conclusively rule out a role for lipoxygenases that can (i) consume NO in the presence of arachidonate, and (ii) generate HPETE that may also be used as a peroxidase substrate for PGHS-2 [28,29]. In additional experiments, COS-7 cells transiently transfected with hPGHS-2 also exhibited HPETE- or H₂O₂-dependent NO consumption (Figures 3 and 4). Here, selective introduction of the gene encoding hPGHS-2 excludes NO consumption by additional peroxidases and confirms the ability of cellular PGHS-2 to consume NO during peroxidase turnover.

In vascular cells, iNOS and PGHS-2 are induced in tandem by the same pro-inflammatory cytokines. Previous studies have shown that these pathways interact at a number of levels, including both stimulation and suppression of PGHS-2-dependent prostaglandin generation by NO [30–33]. Herein, addition of L-arginine to cytokine-stimulated macrophages enabled NO generation by endogenous iNOS that could be completely depleted by HPETE addition (Figure 2C). Indeed, comparison of rates of NO consumption in our study (0.42 ± 0.084 nmol $\cdot$ min⁻¹ $\cdot$ 10⁶ cells⁻¹) with published estimates of NO generation by murine macrophages (0.1–0.2 nmol $\cdot$ min⁻¹ $\cdot$ 10⁶ cells⁻¹) indicate further that PGHS-2 is expressed at levels that can impact significantly on NO levels under pro-inflammatory conditions in mammalian cells, and underscore its potential ability to modulate NO signalling *in vivo* [34,35].

Lipid hydroperoxides are generated by a number of pathways in vascular cells, including (i) PGHS-mediated oxidation of arachidonate to PGG₂, which will be sensitive to aspirin, celecoxib or indomethacin, (ii) lipoxygenase-mediated oxidation of arachidonate to HPETE, and (iii) non-enzymic LDL peroxidation associated with macrophage-derived foam cells. All of these potential sources of PGHS substrate are elevated in vascular disease and inflammation, and could provide substrates for PGHS-2 peroxidase turnover. Indeed, numerous human studies have shown that PGHS-2-selective inhibitors or aspirin can acutely improve NO bioactivity *in vivo* in subjects with vascular disease [8,9,36–40].

In summary, PGHS-2-dependent NO consumption has been demonstrated by isolated enzyme, PGHS-2-transfected COS-7 cells and inflammation-activated murine macrophages, with turnover rates sufficient to fully deplete endogenous iNOS-derived NO. Catalytic NO scavenging by PGHS-2 provides a novel functional interaction between NO and PGHS-2 that may explain how PGHS-2 inhibition improves NO bioactivity *in vitro* in models of inflammation, and in human vascular disease *in vivo* [3,4,8,9].

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