Over-expression of GTP-cyclohydrolase 1 feedback regulatory protein attenuates LPS and cytokine-stimulated nitric oxide production

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Vascular Medicine 2008; **13:** 29–36

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Abstract: GTP-cyclohydrolase 1 (GTP-CH1) catalyses the first and rate-limiting step for the *de novo* production of tetrahydrobiopterin (BH_4), an essential cofactor for nitric oxide synthase (NOS). The GTP-CH1-BH_4 pathway is emerging as an important regulator in a number of pathologies associated with over-production of nitric oxide (NO) and hence a more detailed understanding of this pathway may lead to novel therapeutic targets for the treatment of certain vascular diseases. GTP-CH1 activity can be inhibited by BH_4 through its protein–protein interactions with GTP-CH1 regulatory protein (GFRP), and transcriptional and post-translational modification of both GTP-CH1 and GFRP have been reported in response to proinflammatory stimuli. However, the functional significance of GFRP/GTP-CH1 interactions on NO pathways has not yet been demonstrated. We aimed to investigate whether over-expression of GFRP could affect NO production in living cells. Over-expression of N-terminally Myc-tagged recombinant human GFRP in the murine endothelial cell line sEnd 1 resulted in no significant effect on basal BH_4 nor NO levels but significantly attenuated the rise in BH_4 and NO observed following lipopolysaccharide and cytokine stimulation of cells. This study demonstrates that GFRP can play a direct regulatory role in iNOS-mediated NO synthesis and suggests that the allosteric regulation of GTP-CH1 activity by GFRP may be an important mechanism regulating BH_4 and NO levels *in vivo*.

Key words: cytokines; endothelial cells; lipopolysaccharide; nitric oxide

Introduction

In mammals, 6*R*-L-5,6,7,8-tetrahydrobiopterin (BH_4) is a cofactor for all three isoforms of nitric oxide synthase (NOS)[1] and its synthesis occurs via two distinct pathways: the first is the *de novo* pathway which uses GTP as a substrate in which the rate-limiting enzyme is GTP-cyclohydrolase 1 (GTP-CH1) and the second is the salvage pathway which recycles the intracellular pool of pre-existing dihydropterins.[2,3]

The GTP-CH1-BH_4 pathway is emerging as an important regulator in a number of pathologies associated with over-production of nitric oxide (NO). It is known that induction of the non-constitutively expressed isoform of NOS (inducible iNOS or NOS2) has been implicated in circulatory failure in various models of septic shock.[4–7] *De novo* biosynthesis of BH_4 is up-regulated in response to proinflammatory stimuli[8,9] and pharmacological inhibition of the BH_4 pathway in sepsis models improves survival whilst non-selective NOS inhibition (LNMMA) has no survival benefit.[10] In addition, recent evidence indicates that the GTP-CH1-BH_4 pathway is an important regulator of inflammatory and neuropathic pain.[11] Together, these studies imply that interrupting the availability of BH_4 by modulating the BH_4 biosynthetic pathway may be a useful therapeutic strategy for selectively limiting NO over-production in a number of pathologies.

The regulation of GTP-CH1 activity appears to be the first-line mechanism in the maintenance of BH_4 homeostasis. *In vitro*, regulation of GTP-CH1 activity has been reported at the level of transcription and translation.[9,12] Recombinant enzyme studies have also demonstrated that GTP-CH1 activity can potentially be modified post-translationally via

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 10.1177/1358863X07085916

an allosteric interaction with GTP cyclohydrolase 1 feedback regulatory protein (GFRP).[13]

Increasing evidence suggests that in mammalian cells proinflammatory stimuli co-ordinately regulate GTP-CH1 and GFRP.[14,15] However, to date, the functional significance of the stoichiometry of these two proteins on NO synthesis has not been demonstrated. In the present study, we examined the role of GFRP in the regulation of BH_4 synthesis and NO generation in endothelial cells both basally and following cytokine-stimulated iNOS induction.

Our hypothesis was that the reported reduction in GFRP expression following proinflammatory stimulation[14] is necessary for optimal GTP-CH1 activity, BH_4 production and maximal iNOS activity. To test this hypothesis we have generated cell lines that constitutively over-express GFRP and characterized their BH_4 synthesis and NO production. This study demonstrates, for the first time, that alterations in GFRP levels can significantly impact upon lipopolysaccharide (LPS) + cytokine-stimulated NO production.

Methods

Expression and cloning of N-Myc GFRP

A construct of an N-terminally Myc-tagged form of human GFRP (accession number: NM_005258) was created by PCR amplification using a 5′oligonucleotide primer encoding a *Hin*dIII restriction site followed by the 9E10 N-Myc epitope [GATCAAGCTTACCATGGCCGAACAAAAACTCA TCTCAGAAGAGGATCTG GGCGGCCCCTA CCTGCTCATCAGCACC] and a 3′oligonucleotide primer containing a downstream Xho 1 site [CTGACTCGAGTCACTCCTTGTGCAG ACA CCAC]. Subsequent PCR amplification (94°C, 10 s; 40ºC, 10 s; 72ºC, 30 s; 30 × cycles) yielded a product which was restriction digested (*Hin*dIII and Xho 1) and ligated to similarly digested pcDNA 3.1 Hygro plasmid (Invitrogen) and transformed into *Escherichia coli* (DH5α – Invitrogen). Recombinant plasmids were selected and the insert verified by sequencing (MWG Biotech).

Transfection of sEnd 1 cells

The murine endothelial cell line sEnd 1[16] was used for all studies. Cells were cultured in Dubecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% FBS, 100 units penicillin G Sodium and 100 μg streptomycin sulphate; 2 mM L-glutamine (Invitrogen) in a humidified incubator (37ºC / 5% CO_2). GFRP/pcDNA 3.1 Hygro or the pcDNA 3.1 Hygro (empty vector control) were transfected into the sEnd 1 cells using TFX 20 Transfast transfection reagent (Promega). Selection medium containing 500 μg/ml hygromycin yielded individual hygromycin-resistant colonies which were isolated and expanded. To confirm successful transfection of N-Myc GFRP, adherent cells were lysed in RIPA buffer, and proteins resolved by SDS PAGE and analysed by western blotting using the 9E10 monoclonal anti 9E10 Myc antibody (1:1,000 dilution).

Cytokine stimulation

The effects of GFRP over-expression on NO production were investigated under both basal and proinflammatory conditions. GFRP-transfected and control cells from individual clones were grown to 70% confluency. Media were replaced with either stimulating medium (containing 10 ng/ml tumour necrosis factor α (TNF α), 100 U/ml interferon gamma γ (IFN γ) (R&D Systems) and 5 μg/ml lipopolysaccaride (LPS – *Salmonella typhosa*; Sigma)) or unsupplemented medium (control). At various time points (as indicated) media from each well were removed for the measurement of nitrite.

Greiss assay

The stable breakdown product of NO, nitrite, was measured colorimetrically using the Greiss assay. Briefly, 50 μl of Greiss reagent A (1% sulphanilamide (w/v), 5% phosphoric acid (v/v)) and 50 μl Greiss reagent B (0.1% *N*-(1-naphthyl)ethylenediamine (w/v)) were added to 100 μl of media sample. Absorbance at 560 nM was determined.

iNOS protein expression time course

Cells from two clones (one GFRP over-expressing and one empty vector control) were grown to 70–80% confluence, after which stimulating media were added. Media were subsequently removed at various time points from individual wells and the adherent cells lysed in RIPA buffer and the protein concentration determined. iNOS protein expression post-stimulation was detected using SDS PAGE and western blotting of cell lysates (which were loaded in equal concentrations of total cellular protein), using a polyclonal anti iNOS antibody (1:3,000 dilution) (Insight Technologies).

Intracellular BH_4 measurements

Intracellular BH_4 levels were measured in GFRP and empty vector cell lines ± stimulating medium. Adherent cells were washed in phosphate buffered saline and cells harvested in 100 μl phosphate buffered saline, 10 μl of the suspension was removed for a protein assay and the remainder centrifuged at 14,000 rpm (4°C). The resultant pellet was resuspended in 300 μl of extraction buffer (0.1 M perchloric acid, 6.5 mM 1,4-dithioerythritol and 2.5 mM

Diethylenetriaminepentaacetic acid, DETAPAC) and further centrifuged at 14,000 rpm (4°C). The supernatant was loaded onto an HPLC coupled to an electrochemical detector and intracellular BH_4 levels measured as described previously.[17]

Determination of nitrite source

One GFRP over-expressing clone and one empty vector-transfected clone were treated with either stimulating media or unsupplemented media for 22 hours in the presence of the non-selective NOS inhibitor L-NAME (10 μM) and the iNOS selective inhibitor 1400W (100 nM).

Determination of iNOS mRNA expression

cDNA was prepared from GFRP over-expressing and empty vector control cells pre- and post-LPS + cytokine stimulation. End-point PCR was performed using oligonucleotide primers designed against murine iNOS (accession number NM_010927); [CAC CTT GGA GTT CAC CCA GT] [ACC ACT CGT ACT TGG GAT GC] and PCR amplification was performed (94°C, 10 s; 60°C, 10 s; 72°C, 30 s × 30 cycles).

Superoxide measurement

Superoxide O_2^- formation in GFRP over-expressing and empty vector control cells was assayed with the fluorescent dye dihydroethidine (DHE) (Sigma Aldrich) using flow cytometry. GFRP over-expressing and empty vector control cells were plated at a density of 1×10^6 in 6-well plates and treated ± stimulating media. Cells were then treated with DHE to a final concentration of 10 μM for 30 minutes. Cells were subsequently trypsinized and resuspended in trypsin neutralizing solution. Fluorescence intensities were monitored and recorded using a FACScan cell sorter (BD Biosciences) and data were analyzed using CellQuest Pro Software (BD Biosciences).

Results

Characterization of GFRP-pcDNA 3.1 and pcDNA 3.1 empty vector-transfected cells lines

Consistent with published findings,[14] stimulating media increased GTP-CH1 and decreased GFRP mRNA indicating that these cells were suitable to test our hypothesis (data not shown). Anti-Myc immunoblotting of all cell lines from GFRP-transfected wells showed an immunoreactive band of the predicted size of Myc-GFRP (~12 kDa) with differing levels of N-Myc GFRP between clones, whilst all empty vector-transfected cell lines showed no immunoreactive band (Figure 1).

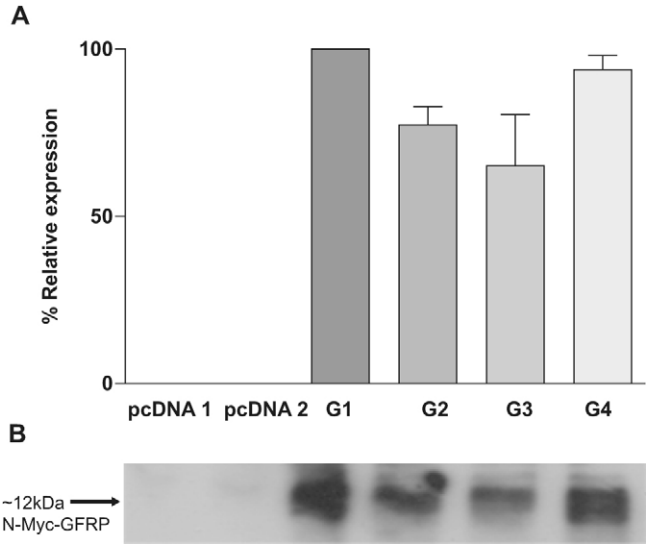

Figure 1 Densitometric analysis and representative blot showing immunoreactive bands obtained from cell lysates probed with 9E10 anti-Myc antibody from empty vector-transfected clones (pcDNA 1 and 2) and N-Myc GFRP-transfected clones (G1–G4). Percentage relative N-Myc GFRP per mg total cellular protein compared to G1. Data represent mean ± SEM ($n = 5$).

Effects of GFRP over-expression on NO synthesis

There was no detectable difference in basal nitrite production between GFRP over-expressing and empty vector cell lines (Figure 2A). Following LPS + cytokine stimulation, nitrite production was significantly increased in all cell lines. However, the magnitude of the increase in nitrite was significantly attenuated in all GFRP-transfected lines when compared with empty vector lines (Figure 2A), and extent of this attenuation inversely correlated with the level of N-Myc-tagged GFRP present in each cell line (Figure 2B). Total NOx (nitrite + nitrate) was also reduced in a similar pattern to nitrite alone (data not shown).

Determination of nitrite source

Addition of L-NAME but not 1400W to unstimulated cells significantly reduced nitrite production indicating that eNOS was the source of nitrite in these cells (Figure 3A). In contrast, both L-NAME and 1400W attenuated the stimulated increase in NO production, indicating that this was due to iNOS induction (Figure 3B).

Effects of GFRP over-expression on iNOS mRNA

iNOS mRNA was readily detectable in cells that had been stimulated with LPS and cytokines and absent in unstimulated cells. There was no observed difference in iNOS mRNA expression between GFRP over-expressing and empty vector control cells (Figure 4A).

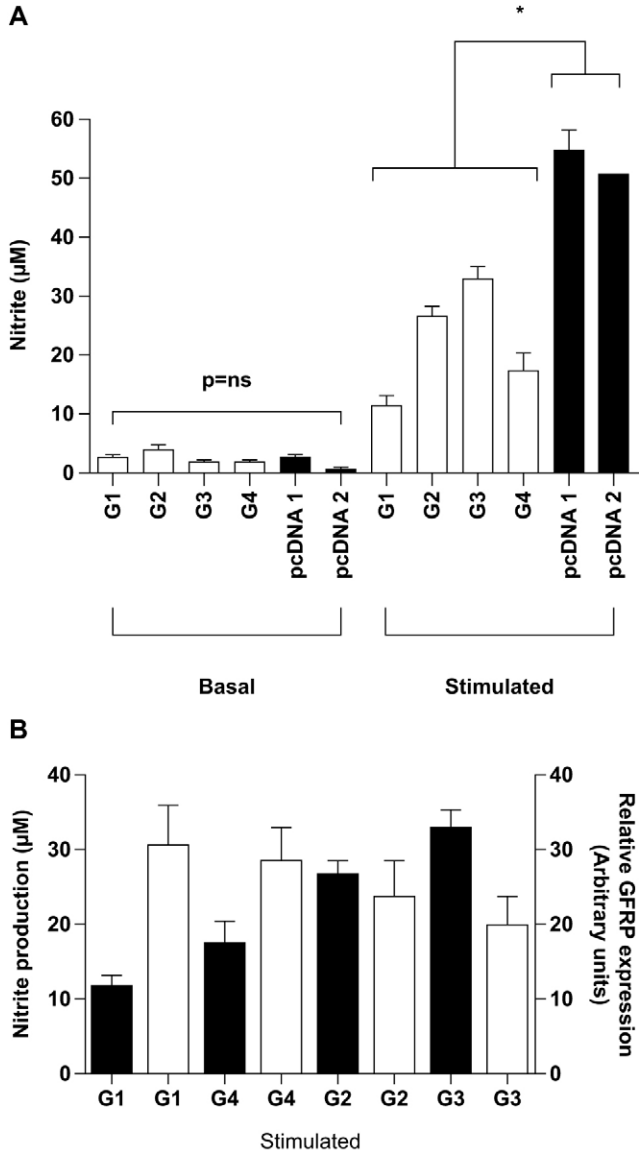

Figure 2 (**A**) Nitrite production from GFRP over-expressing (white bars) and empty vector-transfected (black bars) cells as measured by Greiss assay, both basally and post-cytokine + LPS stimulation. $*p < 0.05$ one-way ANOVA with Tukey Kramer post hoc test. Data represent mean ± SEM ($n = 5$). (**B**) Densitometric analysis of expression levels of N-Myc-tagged GFRP in individual clones G1–G4 (white bars) and inverse correlation with nitrite generation following cytokine + LPS stimulation (black bars). R^2 of mean values = 0.996 ($n = 5$).

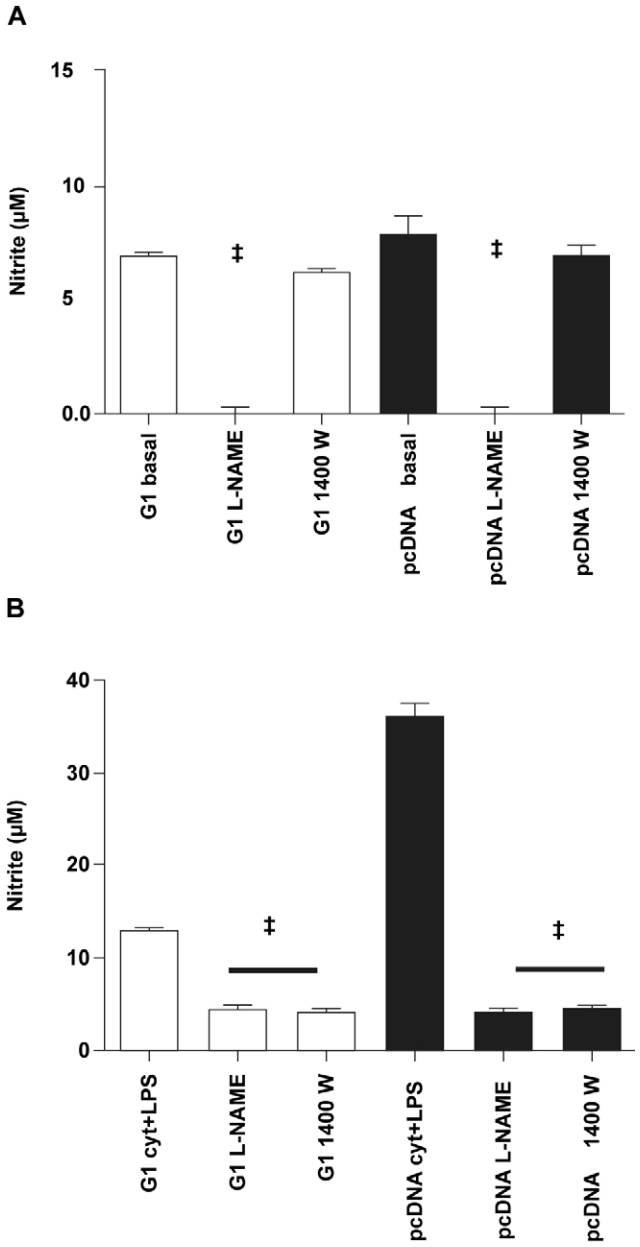

Figure 3 Effect of NOS inhibitors L-NAME and iNOS-specific inhibitor 1400W on nitrite production from mock-transfected (pcDNA 1) and GFRP over-expressing cell line (G1) (**A**) basally; $*p < 0.001$ L-NAME versus basal or 1400W treated and (**B**) following cytokine + LPS stimulation. $*p < 0.05$ basal, L-NAME or 1400W versus LPS + cytokine stimulation alone. Data represent mean ± SEM ($n = 4$).

Time course of NO production

In both the empty vector and GFRP over-expressing lines, the time course of LPS + cytokine stimulated NO production was similar. However, GFRP over-expressing cells had attenuated nitrite production at all time points (Figure 4B).

Effects of GFRP over-expression on iNOS protein

Analysis of iNOS protein levels following cytokine stimulation revealed a marked reduction in cells over-expressing GFRP compared with empty vector control cells. This attenuation occurred throughout the time course of stimulation (Figure 4C).

Intracellular BH_4 content of GFRP over-expressing and empty vector cell lines

Basally there was no significant difference in intracellular BH_4 between GFRP over-expressing and empty vector control cells. Intracellular BH_4 levels were measured at 4 hours post-stimulation – a time at which iNOS protein synthesis commences

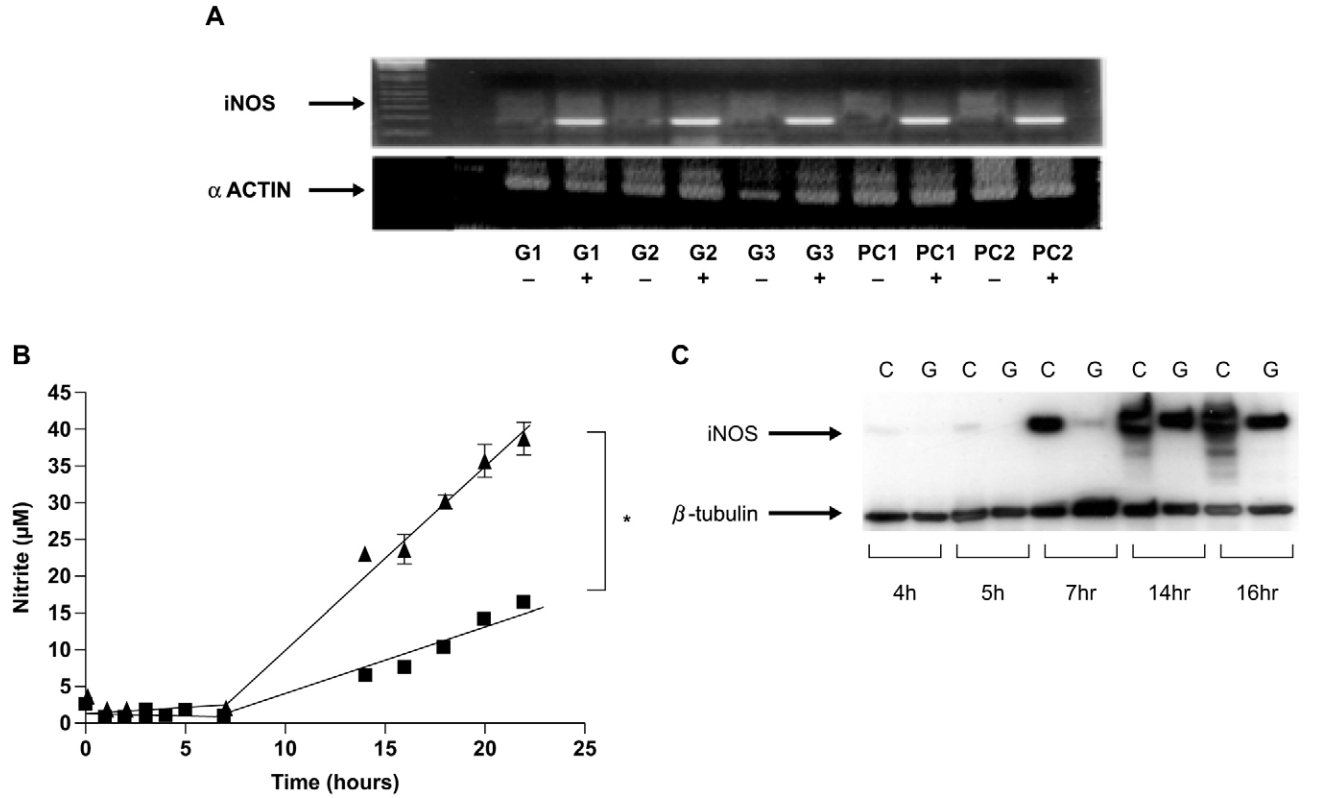

Figure 4 (**A**) PCR amplification of iNOS mRNA in GFRP over-expressing (G1–G3) and empty vector-transfected control cells (PC1 and PC2) pre (–) and post (+) LPS + cytokine stimulation. (**B**) Temporal nitrite accumulation in media following cytokine + LPS stimulation in an empty vector-transfected control (▲) and a GFRP over-expressing cell line (■); $*p < 0.0001$ two-way ANOVA. (**C**) Temporal expression of iNOS protein in GFRP over-expressing cells (G) and empty vector control cells (C). Data represent mean ± SEM ($n = 5$).

(Figure 4C). In control cells, BH_4 levels were doubled 4 hours post-stimulation compared with unstimulated control cells. In contrast, BH_4 levels were not significantly different between stimulated and unstimulated GFRP over-expressing cells (Figure 5).

Superoxide (O_2^-) measurements

To investigate whether GFRP over-expression had any effect on O_2^- generation, we exposed cultured cells to DHE and assayed O_2^- formation by measuring the DHE fluorescence. Results indicate that there was no significant difference in relative fluorescence intensity between GFRP over-expressing and empty vector control cells both basally and post LPS + cytokine stimulation (basal: 103 ± 24 for GFRP vs 128 ± 40 empty vector control cells; stimulated: 195.68 ± 64 for GFRP vs 153.20 ± 53 for pcDNA 3.1 ($n = 4$, p = ns, Student's T test).

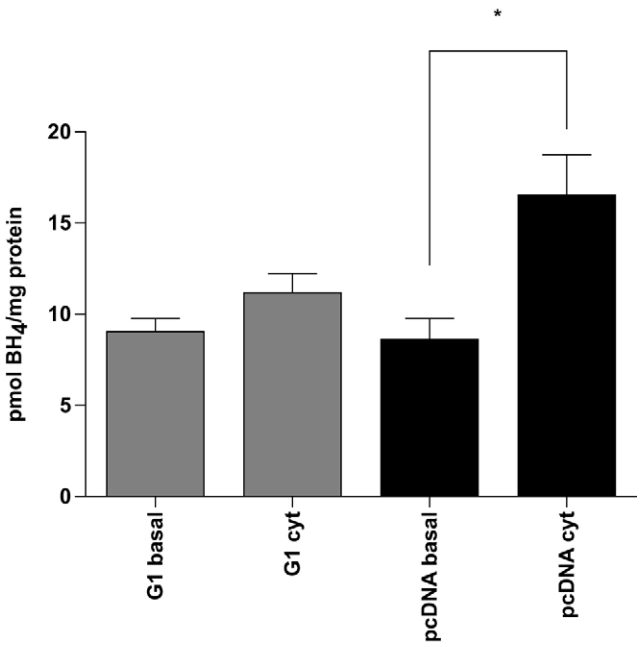

Figure 5 Intracellular BH_4 levels from one GFRP over-expressing cell line (G1) and one empty vector-transfected cell line (pcDNA 1) basally at 4 hours and 4 hours post-cytokine + LPS stimulation. $*p < 0.05$ two-tailed Student's t-test. Data represent mean ± SEM ($n = 3$ in duplicate).

Discussion

It has been reported that proinflammatory stimuli reduce the expression of GFRP *in vitro*[14] whilst GTP-CH1 expression and BH_4 concentration are increased.[14,18–20] This coordinate regulation of

GFRP and GTP-CH1 has been suggested to be required for the generation of an adequate supply of BH_4 which serves as an essential cofactor for iNOS. BH_4 only causes feedback inhibition of GTP-CH1 in the presence of GFRP. Thus, the coordinate regulation of GFRP and GTP-CH1 following proinflammatory stimulation is likely to render GTP-CH1 relatively insensitive to increases in BH_4 and therefore allow maximal BH_4 synthesis. We hypothesized that artificial elevation of GFRP levels in endothelial cells would retain the sensitivity of GTP-CH1 to BH_4-mediated feedback inhibition and would therefore impact upon NO generation.

To test this hypothesis, in the present study we generated stably transfected clones of the murine endothelial cell line sEnd 1 that over-express an N-terminally Myc-tagged form of human GFRP. The sEnd 1 cell line was chosen as a model for studies of GTP-CH1/GFRP/NOS interactions as it is known to express both eNOS, GTP-CH1 and GFRP basally, and iNOS following stimulation[21,22] (and data presented in this study). We used N-terminally tagged human GFRP as modifications at the N-terminus do not affect the interaction with GTP-CH1 or its activity *in vitro*.[23]

Effects of GFRP over-expression in the basal state

In the basal state, over-expression of GFRP had no effect on BH_4 levels and did not alter NO generation from eNOS, suggesting that endogenous GFRP levels are sufficient to saturate GTP-CH1, or that BH_4 levels are not sufficiently high to exert regulation of GTP-CH1 in this situation.

Interestingly, in studies where pharmacological manipulation of BH_4 levels (using BH_4 analogues or inhibitors of the BH_4 biosynthetic pathway) have been used, no effects were observed on eNOS activity in endothelial cells.[21,24,25] This lack of effect might be explained by the tight binding of BH_4 to eNOS in endothelial cells, which prevents BH_4 analogues from displacing BH_4 from eNOS. The eNOS-bound BH_4 is sufficient for optimal eNOS activity under basal conditions, explaining the lack of effect of BH_4 biosynthetic inhibitors. Therefore, limitation of BH_4 availability under basal conditions is unlikely to render eNOS inactive in endothelial cells.

It has been suggested that limitation of BH_4 may result in NOS uncoupling and the subsequent generation of O_2^-.[26] We therefore measured O_2^- in our cell lines to investigate whether or not GFRP over-expression had any effect on O_2^- generation. In support of the lack of effect on basal BH_4 levels, O_2^- levels were found to be not significantly different between GFRP over-expressing and empty vector control cells, suggesting that GFRP over-expression does not cause eNOS to become uncoupled.

Effects of GFRP over-expression following cytokine + LPS stimulation

In contrast to the effects of GFRP over-expression in the basal state, GFRP over-expression attenuated the increase in BH_4 and iNOS-mediated NO production following LPS + cytokine stimulation. The extent of attenuation inversely correlated with the level of N-Myc GFRP expression and is therefore unlikely to be due to clonal variation in the cell lines. Consistent with this, clonal cell lines transfected with empty vector all produced high levels of NO when stimulated with LPS + cytokines. In empty vector control cells, the stimulated increase in BH_4 occurred at the time at which iNOS is first expressed. This is the first demonstration in living cells that altered GFRP levels can impact upon BH_4 and NO biosynthesis.

It is known that basal levels of GTP-CH1 and BH_4 are not adequate for optimal iNOS function,[27,28] hence it has been proposed that transcriptional regulation by proinflammatory cytokines upregulates GTP-CH1 and downregulates GFRP, thereby rendering GTP-CH1 relatively insensitive to changes in BH_4 and resulting in maximal BH_4 biosynthesis for iNOS. Consistent with this, in our control cells, BH_4 levels are significantly elevated following cytokine stimulation compared with unstimulated control cells. In contrast, we observe no such increase in intracellular BH_4 levels in our GFRP over-expressing cells following cytokine + LPS stimulation. Thus, by artificially increasing the GFRP:GTP-CH1 ratio in our over-expressing system, a significant proportion of GTP-CH1 appears to still be sensitive to feedback inhibition by BH_4. Once again, the effect of reduced BH_4 production resulting from GFRP over-expression is consistent with pharmacological manipulation of the BH_4 pathway in stimulated cells.[21,24,25]

This preferential inhibition of iNOS may occur because newly synthesized iNOS requires BH_4 for full activity, whereas an existing pool of eNOS is already tightly bound to BH_4. Additionally, it has been demonstrated that of all three NOS isoforms, iNOS forms the least stable dimers and BH_4 availability seems to be more important for iNOS dimer formation and stabilization compared with the other NOS isoforms.[29] An interesting and unexpected finding from this study is that iNOS protein levels are also reduced in cells over-expressing GFRP and we believe that this effect may be explained by increased proteolysis of unstable iNOS monomers that are formed in the absence of sufficient BH_4.

Interestingly, no significant difference was detected in O_2^- generation post-LPS + cytokine stimulation despite BH_4 levels being lower in GFRP over-expressing cell lines. This may indicate differential effects of BH_4 limitation on the ability to uncouple iNOS compared with eNOS or it is possible that BH_4-deficient iNOS is not capable of O_2^- production in our cell lines. However, our observation that reduced BH_4 synthesis in GFRP over-expressing cell lines is associated with decreased accumulation of iNOS protein, may imply that iNOS is not rendered uncoupled but is merely degraded more rapidly.

We postulate that the observed effects of GFRP over-expression on stimulated NO generation stem from a destabilized iNOS protein and not as a result of changes in iNOS mRNA expression. In support of this, we detected no differences in iNOS mRNA expression levels between stimulated GFRP over-expressing and empty vector control cells. The mechanism of the reduced iNOS protein observed in the presence of GFRP over-expression warrants further investigation. The pathological significance of this finding is that in situations where overproduction of NO from iNOS is implicated in disease progression, interventions that limit BH_4 synthesis will result in attenuation of iNOS protein accumulation and NO production.

The induction of iNOS following a proinflammatory insult is believed to occur primarily as an anti microbial defence mechanism[30] and this data would imply that a destabilization of iNOS homodimers in macrophages would reduce the beneficial antimicrobial effects of NO. Conversely, the induction of iNOS in smooth muscle cells is believed to contribute to profound vasodilatation resulting in hypotension and cardiovascular collapse; in this setting, destabilization of iNOS homodimers would be beneficial and potentially limit the extent of hypotension observed.

In contrast to our observations and the published results of pharmacological manipulation of the BH_4 synthetic pathway, Kalivendi, *et al.*[31] have reported that the transient transfection of GFRP in HAEC and COS7 cells results in complete inhibition of BH_4 synthesis under non-stimulatory conditions. These differences might relate to methodological differences between the studies: Kalivendi, *et al.* used transient transfection to over-express GFRP in cultured cells and apparently achieved 100% transfection of both HAEC and COS7. This approach might have resulted in a very high level of over-expression of GFRP and hence complete inhibition of BH_4. Unfortunately, the effects of transient GFRP over-expression on NO production in both the basal state and LPS stimulated state was not reported in the study by Kalivendi, *et al.* and hence a direct comparison with our present study is not possible.

BH_4 inhibition has been shown to improve survival in a rat model of septic shock[10] and reduces pain hypersensitivity in models of neuropathic and inflammatory pain.[11] The implication of our findings is that over-expression of GFRP or manipulation of GTP-CH1 activity by targeting these protein–protein interactions may achieve a similar effect. In addition, a haplotype of the GTP-CH1 gene has been associated with reduced pain following discectomy in humans.[11] Similarly, these data suggest that genetic or environmental factors that alter the expression of GFRP have the potential to influence BH_4 and NO levels, particularly under inflammatory conditions *in vivo*.

In summary, this is the first demonstration that alteration of endogenous GFRP levels and hence altering the GTP-CH1:GFRP ratio plays a regulatory role in BH_4 biosynthesis and iNOS activity and identifies GFRP-GTP-CH1 interactions as a critical regulator of iNOS mediated generation.

Acknowledgements

This work was supported by the Medical Research Council and the British Heart Foundation. The authors would like to thank Professor Steven Gross and Dr Mark Crabtree for their help with this paper. The authors would also like to thank Dr Simon Heales for the use of the BH_4 detection system.

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