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Tetrahydrobiopterin Modulates Cyclooxygenase-2 Expression in Human Mesangial Cells

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Tetrahydrobiopterin (BH_4) biosynthetic pathways are stimulated under inflammatory conditions. The newly synthesized BH_4 serves as a cofactor for optimal activity of inducible nitric oxide synthase (NOS2). In human mesangial cells (HMC), BH_4 is also a limiting factor for NOS2 expression. In this study we show that BH_4 availability can also play a modulatory role in the expression of cyclooxygenase 2 (COX-2) in HMC. Supplementing HMC with the BH_4 donor sepiapterin potentiated IL-1β/TNF-α-induced COX-2 expression by approximately 2-fold. This effect was abolished by methotrexate. In contrast, the NOS inhibitor L-NAME and the soluble guanylate cyclase inhibitor ODQ did not block sepiapterin amplification of COX-2 expression. Moreover, sepiapterin was found to modulate the tyrosine phosphorylation of several cellular substrates, an early event which occurred well before the induction of NOS2 could be evidenced. These findings suggest a role for BH_4 in the modulation of mesangial cell responses to pro-inflammatory stimuli.

Mesangial cells (MC) are specialized smooth muscle cells which line the capillaries of the renal glomerulus where they serve functions of structural support and regulation of glomerular filtration rate. During glomerular inflammation, activated MC generate increased amounts of cytokines, prostanoids, and nitric oxide (NO). Prostanoid synthesis can be controlled at two levels, the release of arachidonic acid from membrane phospholipids, which is catalyzed by phospholipases A_2 (1), and the conversion of arachidonic acid to prostanoids, catalyzed by the rate-limiting enzyme cyclooxygenase (COX). A large body of evidences indicates that up-regulation of the inducible cyclooxygenase (COX-2) is involved in the overproduction of prostanoids associated with inflammatory situations (2, 3). Increased generation of NO by MC is due to the expression of inducible nitric oxide synthase (NOS2) which occurs upon exposure to combinations of cytokines or of cytokines plus lipopolysaccharide (4, 5).

The biochemical pathways leading to the induction of NOS2 and COX-2 in inflammation seem to be intimately related. In fact, a coinduction of the two enzymes has been frequently evidenced, both in inflammatory situations such as glomerulonephritis, rheumatoid arthritis or endotoxemia (6), and under experimental conditions (7). In addition, the products of the two enzymes, NO and prostaglandins (PGs), have been reported to influence the expression of both NOS2 and COX-2 (8-10). BH_4, an essential cofactor for NOS, is a limiting factor for cytokine-induced NO generation in MC (11). The biosynthetic pathways leading to BH_4 generation are activated by pro-inflammatory stimuli in various cell types, including mesangial, smooth muscle and endothelial cells (12-14). In addition, a stimulation of pteridine synthesis is known to occur in patients with diseases involving cell-mediated immunity (15). These observations suggest that BH_4 biosynthesis may be an important process in the immune response. We have recently showed that, in human MC (HMC), BH_4 availability plays a modulatory role in NOS2 protein and mRNA expression (5). Inhibition of *de novo* synthesis of BH_4 during treatment of HMC with a combination of interleukin 1β plus tumor necrosis factor α (IL-1β/TNF-α) resulted in reduced NOS2 expression, while supplementation with the BH_4 donor, sepiapterin, induced a clear potentiation of cytokine-elicited NOS2

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Abbreviations used: HMC, human mesangial cells; PG, prostaglandin; COX, cyclooxygenase; IL, interleukin; TNF, tumor necrosis factor; NOS, nitric oxide synthase; BH_4, 5,6,7,8-tetrahydrobiopterin; DAHP, 2,4-diamino-6-hydroxypyrimidine; L-NAME, L-nitroarginine methyl ester; ODQ, [1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one].

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expression. In this study, we have explored whether IL-1β/TNF-α-elicited expression of COX-2 in HMC could be also modulated in response to changes in BH_4 availability.

MATERIALS AND METHODS

Materials. Recombinant human TNF-α (9.8×10^6 U/mg) was a gift from Knoll Pharmaceuticals (Whippany, NJ). Recombinant human IL-1β (5×10^7 U/mg) was from Boehringer Mannheim (Germany). Cell culture media and fetal calf serum were from Bio-Whittaker. Deoxycytidine 5′-triphosphate [α-^{32}P] (3000 Ci/mmol) was from Amersham (Buckinghamshire, UK). Antibodies for immunoblotting were polyclonal rabbit anti-PGHS 2 (Oxford Biomedical Research, Oxford, MI), mouse monoclonal anti-NOS2 (Affinity Research Products, Nottingham, UK), antiphosphotyrosine antibody PY20 (Santa Cruz Biotechnology, Santa Cruz,CA) and peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulins (Dako, Glostrup, Denmark). The reagents for PGE_2 extraction were from Merck (HPLC grade) and the prostaglandin E_2 enzymeimmunoassay (EIA) system was from Amersham. L-Sepiapterin and [1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one] (ODQ) were from Alexis Co. (Laüfelfingen, Switzerland). All other reagents used were of the highest purity available from Sigma Chemical Co. (St. Louis, MO).

Cell culture. Human mesangial cells (HMC) were obtained and characterized as previously described (4, 5). The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 24 μg/ml gentamycin. For experiments, passages 7 to 12 were used. Confluent HMC were incubated in RPMI without phenol red with the indicated agents in the absence of serum. TNF-α was used at 100 ng/ml and IL-1β was used at 2 ng/ml, final concentrations. Potential toxicity of the reagents used on HMC was evaluated by trypan blue exclusion. According to these criteria, cell viability was above 90% under all experimental conditions studied.

Cyclooxygenase assay. COX activity was measured in whole cells as previously described (16). Briefly, cells were incubated with the various agents for 24 h. During the last hour of incubation 10 μM arachidonic acid was added. Then, the supernatant was collected and assayed for PGE_2.

PGE_2 assay. Cell supernatants (0.5 ml) were acidified with 50% acetic acid (50 μl) and applied to Sep Pak C18 cartridges (Waters Associates, Milford, MA) pre-activated with ethanol and distilled water. Cartridges were then washed with distilled water and petroleum benzine and PGE_2 was eluted with 4 ml of methylformate. The eluted PGE_2 was then dried, reconstituted and measured by enzymeimmunoassay (EIA). The recovery rate for the extraction procedure was 85%, as determined by the addition of synthetic PGE_2 to conditioned medium. The amounts of PGE_2 in cell supernatants were corrected by the total amount of protein in the corresponding cell extracts.

Nitrite determination. The accumulation of nitrite in the cell culture supernatant of HMC was determined by the Griess reaction as previously described (5).

RNA isolation and Northern blot analysis. Total cellular RNA was isolated from HMC using the guanidinium thiocyanate-phenol-chloroform method (17). 10 μg of total RNA were separated on 1% agarose/0.68 M formaldehyde gels, transferred to MSI magnagraph hybridization membranes (Westborough, MA) and UV cross-linked using a UV Stratalinker 1800 from Stratagene (La Jolla, CA). A 1.8 Kb fragment containing the ORF of the human cyclooxygenase type 2 cDNA, gift of Dr. T. Hla (18), was labeled with [α-^{32}P] dCTP using the kit *Rediprime* for random primer labeling from Amersham, and used as a probe for Northern analysis. Hybridization was performed at 42°C for 16 h. Membranes were then washed at final stringency conditions of 1× SSC, 0.5 % SDS at 42°C and exposed to X-OMAT S Kodak film at −80°C using two intensifying screens. In order to ensure even loading of the samples, blots were stripped and rehybridized with a probe derived from a 115 bp cDNA fragment of the human 28 S rRNA gene (pTRI-RNA-28S) from Ambion (Austin, TX). Densitometric analysis was performed using a Vista Scanner UMAX (Umax Data Systems Inc., Taiwan) with the public domain software NIH IMAGE 1.55. Results were calculated as the ratio of COX-2/28 S rRNA expression.

SDS-PAGE and immunoblotting. For analysis of COX-2 or NOS2 expression, HMC were harvested by treatment with trypsin-EDTA. For analysis of protein tyrosine phosphorylation, HMC were washed twice with ice-cold phosphate buffered saline and scraped in the same buffer. HMC were homogenized in 50 mM TRIS-HCl pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM β-mercaptoethanol, 0.2 mM sodium vanadate, 50 mM sodium fluoride, containing 2 μg/ml of each of the protease inhibitors: leupeptin, pepstatin, trypsin inhibitor and aprotinin. Protein concentration was determined by a Bradford Protein Assay (Bio-Rad, Munich, Germany). 8 μg of protein from each experimental condition were electrophoresed on 8% polyacrylamide gels and transferred to Hybond-PVDF membranes from Amersham. Blots were probed either with anti-PGHS-2 (1:200 dilution), anti-NOS2 (1:1000) or anti-phosphotyrosine (1:1000) antibodies followed by secondary antibodies at 1:2000 dilution, and the proteins of interest were visualized using an enhanced chemiluminiscence detection system from Amersham.

RESULTS

Treatment of HMC with a combination of IL-1β plus TNF-α (IL-1β/TNF-α) for 24 h resulted in an increase in cyclooxygenase activity, as indicated by the accumulation of PGE_2 in the incubation medium (Fig. 1A). Supplementing HMC with sepiapterin, which generates BH_4 intracellularly via the dihydrofolate reductase-dependent pterin salvage pathway (19), resulted in a 2-fold potentiation of cytokine-induced PGE_2 release. When the expression of COX-2 was studied at the mRNA level, it was observed that IL-1β/TNF-α elicited the appearance of the COX-2 transcript, which was undetectable in unstimulated cells (Fig. 1B). Addition of sepiapterin ampified COX-2 mRNA expression by approximately two-fold. Sepiapterin alone, did not elicit PGE_2 release, nor COX-2 mRNA induction.

These observations suggested that the availability of BH_4 during HMC activation could be a limiting factor for the expression of COX-2 in this cell type. To test this possibility we explored the levels of COX-2 protein in the presence of inhibitors of BH_4 biosynthetic pathways, under the conditions reported to result in the modulation NOS2 expression (5). 2,4-Diamino-6-hydroxypyrimidine (DAHP), is a widely used inhibitor of GTP-cyclohydrolase I, the rate-limiting enzyme in the *de novo* synthesis of BH_4. Treatment of HMC with DAHP brought about a reduction in cytokine-induced COX-2 protein levels of approximately 30%, but did not affect sepiapterin potentiation of COX-2 expression (Fig. 2). In contrast, methotrexate, an inhibitor of dihydrofolate reductase (19), completely prevented the potentiating effect of sepiapterin. In fact, the combination of sepiapterin plus methotrexate displayed an inhibi-

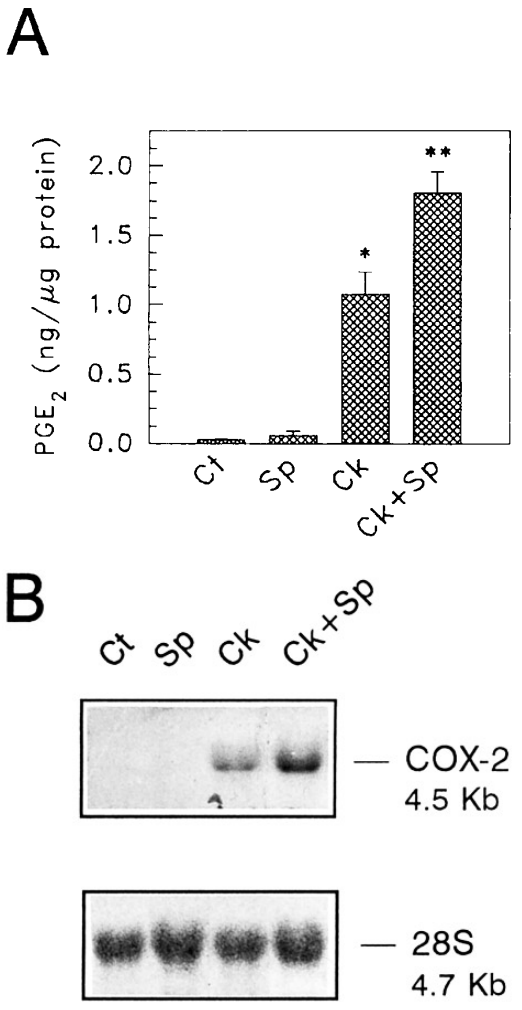

FIG. 1. Effect of sepiapterin on PGE_2 release and COX-2 mRNA expression in HMC. HMC were treated with vehicle (Ct) or 100 μM sepiapterin (Sp), in the presence or absence of a combination of 2 ng/ml IL-1β plus 100 ng/ml TNF-α (Ck), as indicated. (A) The accumulation of PGE_2 in the cell culture supernatant after 24 h was determined by an enzyme-immunoassay. Results are average values ± S.D. of duplicate determinations from four different plates per experimental condition. * $p<0.05$ vs Ct, ** $p<0.05$ vs Ct and vs Ck by t-test. (B) Levels of COX-2 mRNA and of 28S rRNA after a 12 h treatment were determined by Northern blot. Exposures are representative of three independent experiments.

tory effect on the expression of COX-2 elicited by cytokines (Fig. 2).

A role for NO in the amplification of the expression of COX-2 in response to IL-1 has been proposed in rat MC (10). Thus, experiments were designed to evaluate a potential contribution of NO generation to the effect of BH_4. Incubation of HMC with the NOS inhibitor L-NAME during treatment with cytokines resulted in lower levels of COX-2 protein (Fig. 3). However, L-NAME did not reduce significantly sepiapterin potentiation of COX-2 induction. Under these conditions, L-

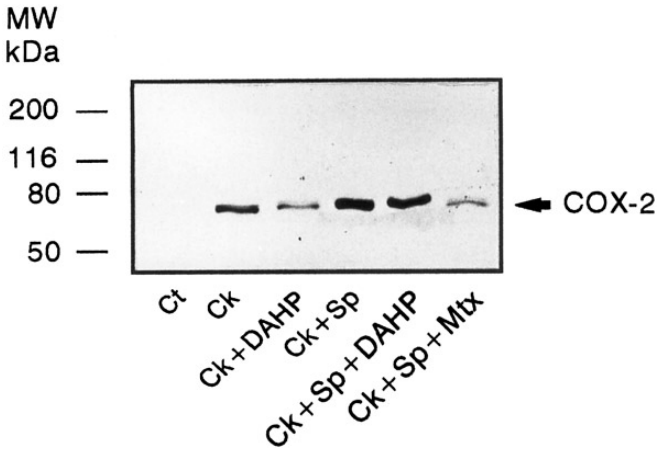

FIG. 2. Effect of donors and inhibitors of BH_4 generation on IL-1β/TNF-α-induced COX-2 protein expression. HMC were treated with the indicated agents for 24 h and the levels of COX-2 protein were estimated by Western blot analysis as detailed in Methods. Ct, control = vehicle; Sp, 100 μM sepiapterin; Ck, cytokine combination: 2 ng/ml IL-1β + 100 ng/ml TNF-α; DAHP, 1.5 mM DAHP; Mtx, 10 μM methotrexate. Results presented are representative of 3 independent experiments. The position of COX-2 is indicated on the right and that of molecular weight markers on the left of the figure.

NAME inhibition of NO generation, estimated from the accumulation of nitrite in HMC medium, was approximately 90%, both in the presence and in the absence of sepiapterin. Since NO amplification of COX-2 expression has been reported to be dependent on cGMP generation (10), we studied the effect of ODQ, an inhibitor of soluble guanylate cyclase, on COX-2 protein levels (Fig. 3). ODQ either did not affect or reduced cytokine-

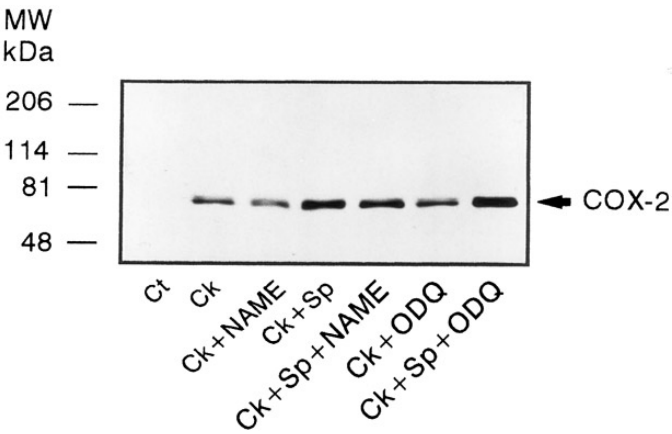

FIG. 3. Effect of L-NAME and ODQ on COX-2 protein expression elicited by cytokines or by cytokines plus sepiapterin. HMC were treated with the indicated agents for 24 h and the levels of COX-2 protein were estimated by Western blot. Ct, control = vehicle; Sp, 100 μM sepiapterin; Ck, IL-1β/TNF-α; NAME, 500 μM L-NAME; ODQ, 0.1 μM ODQ. Results are representative of 3 independent experiments.

induced COX-2 expression slightly (n=5). However, the effect of sepiapterin was not affected. These results suggest that BH_4 potentiation of COX-2 expression could occur, at least in part, by mechanisms independent form the L-arginine-NO-cGMP pathway.

We then tried to identify events in IL-1β/TNF-α signalling pathways which could be susceptible to modulation by sepiapterin supplementation. Tyrosine phosphorylation has been reported to be necessary for COX-2 expression elicited by IL-1β and TNF-α in several experimental systems, including MC, although the proteins implicated have not been fully characterized (20, 21). As it can be appreciated in Fig. 4, extracts of untreated HMC presented several tyrosine phosphopeptides of apparent molecular weights of 50, 70 and 110-130 kDa (Fig 4A). Treatment with sepiapterin alone or with IL-1β/TNF-α increased the tyrosine phosphorylation of the 110-130 kDa group of bands, while treatment with cytokines plus sepiapterin resulted in a higher level of phosphorylation than with either agent alone. Increased phosphorylation could be detected as early as 5 to 15 min after treatment with the stimuli. The presence of methotrexate moderately decreased the level of tyrosine phosphorylation of the 110-130 kDa bands in cells treated with sepiapterin plus cytokines. Western blot analysis of extracts from cells treated for 1 h with the aforementioned stimuli showed no detectable NOS2 or COX-2 protein (results not shown).

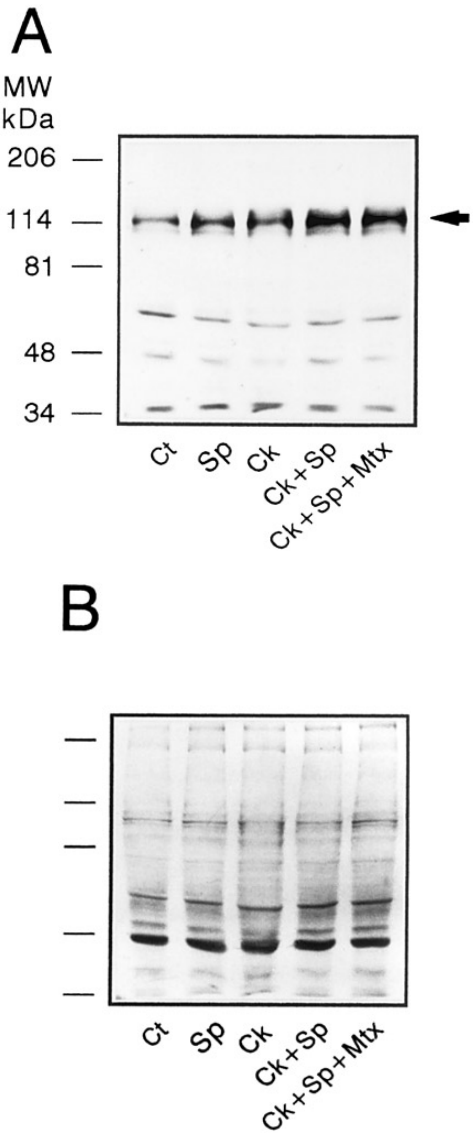

FIG. 4. Effect of cytokines and sepiapterin on tyrosine phosphorylation in HMC. HMC were treated with the indicated agents for 15 min. (A) Detection of tyrosine phosphorylated proteins was performed by Western blot as described in Methods. The position of the 110–130 kDa phosphorylated polypeptides is indicated on the right and that of molecular weight markers on the left of the figure. (B) Coomassie staining of the blot shown in A. Ct, control = vehicle; Sp, 100 μM sepiapterin; Ck, cytokine combination: 2 ng/ml IL-1β + 100 ng/ml TNF-α; Mtx, 10 μM methotrexate. Results are representative of 3 experiments.

DISCUSSION

The observations herein reported suggest that cytokine-induced COX-2 expression, may be modulated by variations in BH_4 supply in HMC, since inhibition of BH_4 biosynthesis reduced, while the BH_4 donor sepiapterin amplified, COX-2 expression. The effect of sepiapterin was abolished by blocking its conversion to BH_4 with methotrexate. Under these latter conditions an inhibition of COX-2 expression was actually observed. This effect could be attributed to a direct inhibitory action of sepiapterin on GTP cyclohydrolase I, which can be evidenced when sepiapterin conversion to BH_4 is blocked, as it has been previously discussed (5). Levels of BH_4 have been reported to increase in the kidneys of rats during experimental endotoxin shock (22). Therefore, the findings reported here could be of relevance under *in vivo* situations.

Cross-talk between COX and NOS pathways ocurrs at multiple levels. NO-related products have been reported to activate COX enzymes (6, 23). In addition, NO has been postulated to amplify IL-1-induced COX-2 expression in rat MC by a mechanism dependent on cGMP generation (10). In turn, prostanoids have been shown to modulate NOS2 expression (9). The observation that BH_4 can potentiate COX-2 expresssion may represent yet another level of interaction between NOS and COX pathways. The fact that inhibitors of NOS or of soluble guanylate cyclase moderately reduced cytokine-induced COX-2 expression suggests that the integrity of the NO/cGMP pathway may be important for COX-2 induction. However, the potentiation exerted by sepiapterin was not influenced by L-NAME or ODQ. This suggests that the effect of sepiapterin does not depend exclusively on NO generation.

BH_4 has been reported to interfere with signal transduction events in several experimental systems. In neurons, BH_4 has been shown to activate calcium currents, and this effect is stereospecific for the 6R isomer (24). It has also been reported that BH_4 can interfere with G protein-mediated pathways in *Dictyostelium* (25). In addition, a role for BH_4 in the modulation of the generation of reactive oxygen species has been postulated (26). The signal transduction cascades activated by IL-1β and TNF-α are not completely understood. However, it is known that both cytokines induce the activation of a $pp60^{src}$-like tyrosine kinase (27) and the tyrosine phosphorylation of several proteins, including the members of the MAP kinase family ERK2 and JNK (28, 29). In addition, the expression of COX-2 has been shown to depend on tyrosine phosphorylation. Inhibitors of tyrosine kinases block COX-2 induction by various stimuli (20, 21), whereas tyrosine phosphatase inhibitors have a positive effect on COX-2 expression (21). In this study, we show for the first time that sepiapterin supplementation can lead to an increase in the tyrosine phosphorylation of several polypeptides in HMC. Although the effect of sepiapterin *per se* does not result in detectable COX-2 expression, it could presumably contribute to the induction of COX-2 by IL-1β/TNF-α. The most prominently phosphorylated bands after exposure to sepiapterin were in the 110-130 kDa molecular weight range. A similar pattern of tyrosine phosphorylation has been observed in MC in response to diverse stimuli, including oxidative stress (30) and cell stretching (31). One of the proteins which results phosphorylated under stretching conditions has been identified as the focal adhesion kinase $pp125^{fak}$ (31). This kinase has been shown to become tyrosine-phosphorylated upon IL-1β stimulation, and to play a role in the elevation of intracellular calcium levels in response to IL-1β (32). The potential involvement of this and other kinases in sepiapterin-mediated modulation of tyrosine phosphorylation should be further explored in order to establish whether there is a causal link between sepiapterin effects on tyrosine phosphorylation and on COX-2 expression.

In summary, we have observed that BH_4 can modulate COX-2 expression and tyrosine phosphorylation in HMC. These findings unravel novel roles for BH_4 on mesangial cell activation and add further evidence in support of a general modulatory role of pteridines in diverse cellular functions.

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