Regulation of Cyclooxygenase by the Heme-Heme Oxygenase System in Microvessel Endothelial Cells

ASIFA HAIDER, RAFAŁ OLSZANECKI, RICHARD GRYGLEWSKI, MICHAL L. SCHWARTZMAN, ELIAS LIANOS, ATTALLAH KAPPAS, ALBERTO NASJLETTI, and NADER G. ABRAHAM

New York Medical College, Department of Pharmacology, Valhalla, New York (A.H., M.L.S., A.N., N.G.A.); Jagiellonian University, Medical College, Department of Pharmacology, Cracow, Poland (R.O., R.G.); Robert Wood Johnson Medical School, New Brunswick, New Jersey (E.L.); and The Rockefeller University, New York, New York (A.K.)

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ABSTRACT
Heme oxygenase (HO) is a microsomal enzyme that oxidatively cleaves heme to form biliverdin, with the release of iron and carbon monoxide (CO). HO not only controls the availability of heme for the synthesis of heme proteins but also is responsible for the generation of CO, which binds to the heme moiety of heme proteins thus affecting their enzymatic activity. Cyclooxygenase (COX) is a heme protein that catalyzes the conversion of arachidonic acid to prostaglandin H$_2$, the precursor of prostanoids that participate in the regulation of vascular function. The goal of the present study was to determine whether the heme-HO system regulates COX enzyme expression and activity in vascular endothelial cells. Endothelial cells stably transfected with the human HO-1 cDNA exhibited a severalfold increase in HO-1 mRNA levels, which was accompanied by an increase in HO activity and a marked decrease in prostaglandin (PG) E$_2$ and 6-keto-PGF$_{1\alpha}$ levels. Exposure of cells to CoCl$_2$, an inducer of HO-1 gene expression, resulted in increases in HO-1 protein levels and HO activity. The increase in HO activity was associated with a subsequent decrease in COX activity, which returned to normal levels following normalization of HO activity. The addition of heme resulted in an increase in COX activity with an increase in PGE$_2$ and 6-keto-PGF$_{1\alpha}$ levels. The degree of HO-1 expression and, consequently, the level of cellular heme, were directly related to COX activity. These results demonstrate that the heme-HO system can function as a cellular regulator of the expression of vascular COX, thus influencing the generation of prostanoids, PGE$_2$ and PGl$_2$, known to play a role in vascular homeostasis.

Cyclooxygenase(s) catalyzes the oxygenation and peroxidation of arachidonic acid to prostaglandin endoperoxide H$_2$, the immediate precursor of prostaglandins and thromboxane. Two COX isoforms encoded by two related genes have been identified; COX-1 is constitutively expressed and is considered to generate prostaglandins for normal physiological functions whereas COX-2 is, in most tissues, an inducible enzyme expressing rapidly and transiently in response to a variety of stimuli (Smith et al., 1996). Both COX isoforms are heme proteins (van der Ouderaa et al., 1979). Heme binds to the COX apoenzyme with a stoichiometry of approximately one heme molecule per each subunit (Smith and Marnett, 1991). It is well documented that the heme prosthetic group of COX is essential for the expression of catalytic activity (Smith and Marnett, 1991). Accordingly, the possibility arises that variations in the cellular levels of heme impact on the amount of catalytically active COX present in cells. The cellular level of heme is regulated by the rate of its synthesis and degradation. Heme degradation occurs almost exclusively by oxidative cleavage of the ε-meso carbon bridge of heme, eventually leading to the formation of equimolar amounts of biliverdin, iron, and CO. The heme oxygenase (HO) system controls the rate-limiting step in heme degradation. To date, three HO isoforms (HO-1, HO-2, and HO-3) encoded by different genes have been identified (McCoubrey et al., 1992, 1997; Shibahara et al., 1993). HO-1 is a 32-kDa heat shock protein (Shibahara et al., 1987; Mitani et al., 1989), which is inducible by numerous stimuli (Abraham et al., 1996). HO-2 is a constitutively synthesized 36-kDa protein, which is abundant in the brain and testis (McCoubrey et al., 1992, 1997). HO-3 is related to HO-2 but is the product of a different gene, and its ability to catalyze heme degradation is much lower than HO-1 (McCoubrey et al., 1997).

ABBREVIATIONS: COX, cyclooxygenase; HO, heme oxygenase; RCME cells, rabbit coronary microvessel endothelial cells; PG, prostaglandin; BAEC, bovine aortic endothelial cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TTBS, Tween 20/Tris-buffered saline; EIA, enzyme-linked immunoassay; SnMSP, Sn-mesoporphyrin; RCME-HHO-1, RCME transfected with the human HO-1 cDNA; HHO-1, human HO-1; NS398, N-(2-cyclohexyloxy)-4-nitrophenoxy)methanesulfonamide.
The goal of the present study was to determine whether interventions that alter cellular heme levels influence COX enzyme expression and activity. Previously we established a model of rabbit coronary microvessel endothelial cells (RCME cells) overexpressing the human HO-1 gene (Abraham et al., 1995). We used these cells, as well as endothelial cells in which HO activity was modulated by chemical inducers, and measured the expression of COX isoforms and the generation of COX-derived prostaglandins (PG). The results demonstrate that the heme-HO system may function as a cellular regulator of the expression of vascular COX, thus influencing the generation of prostanoids, PGE\(_2\) and PGI\(_2\), known to play a role in vascular homeostasis.

Materials and Methods

Vascular Endothelial Cell Cultures. RCME cells were isolated from the mid-portion of the rabbit myocardium by collagenase digestion, filtration, and centrifugation as described by Gerritsen et al. (1988). The cells were seeded onto fibronectin-coated six-well culture plates and incubated in Dulbecco’s modified Eagle’s medium containing 20% plasma-derived serum, endothelial cell growth factor at 100 ng/ml, Carlsbad, CA) that was supplemented with fetal bovine serum (CLONTECH, Palo Alto, CA). The blots were washed three times in PBS, 0.1% Tween, 0.05% SDS, and 37°C. Cells were used in the experiments (passages 12–25) were cultured in Dulbecco’s modified Eagle’s medium and 10% fetal calf serum supplemented with 20 mM Hepes, 0.35 mM L-glutamine, and 0.06 mg/ml gentamicin. These cells were used to overexpress the human HO-1 gene by transfection and clonal selection (Abraham et al., 1995). Cells expressing the human HO-1 gene, referred to as RCME-HHO-1 cells, were grown in parallel with control RCME cells as previously described (Abraham et al., 1995).

Bovine aortic endothelial cells (BAEC) were obtained from bovine thoracic aortae after treatment with 0.5% (w/v) trypsin and 0.02% EDTA (Makarski, 1981). The cells were pooled and seeded in T25 flasks precoated with 1% gelatin. After incubation for 1 h at 37°C in 5% CO\(_2\), nonadherent cells were removed. BAEC were cultured at 37°C in humidified air containing 5% CO\(_2\) in Opti-MEM I (Invitrogen, Carlsbad, CA) that was supplemented with fetal bovine serum (5%) and the following antibiotics: penicillin G sodium salt (100 units/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (0.25 μg/ml). Upon reaching confluence, cultured endothelial cells were passaged and reseded into T75 culture flasks. Cells were cultured in a serum-deprived medium (0.5% fetal bovine serum) for 24 h before addition of heme (10 μM) for an additional 24 h in culture medium containing 10% fetal bovine serum. Endothelial cells were identified by their typical cobblestone morphology and by positive staining for factor VIII-related antigen.

Northern Analysis. Total RNA was isolated either by the guanidinium thiocyanate-phenol extraction method or using TRIzol reagent (Invitrogen) following the instructions provided by the manufacturer. Total RNA (10 μg per lane) was denatured, electrophoresed on 1.2% agarose formaldehyde gels, transferred to a positively charged nylon membrane (Hybond N++; Amersham Pharmacia Biotech, Piscataway, NJ) and UV cross-linked (Stratalinker; Stratagene, La Jolla, CA). Membranes were prehybridized for 1 to 2 h at 60°C and subsequently hybridized overnight at 60°C with random primer [\(^{32}P\)]dCTP-labeled human HO-1 cDNA, rat HO-1 cDNA, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (CLONTECH, Palo Alto, CA). The blots were washed three times with a solution containing 0.5% bovine serum albumin, 5% SDS, and 1 mM EDTA in 0.2 × standard saline citrate at 56 to 60°C and then exposed to X-ray film at −80°C.

Immunoblot Analysis. Cells were incubated with stimulants in T75 flasks for 24 h; they were then washed with phosphate-buffered saline and trypsinized [0.05% trypsin (w/v) with 0.02% EDTA]. The pellets were lysed in buffer [50 mM Tris-Cl, 10 mM EDTA, 1% Triton X-100 (v/v), 1% PMSF, 0.05 mM pepstatin A, and 0.2 mM leupeptin] and after mixing with sample loading buffer [50 mM Tris-Cl, 10% SDS (w/v), 10% glycerol (v/v), 10% 2-mercaptoethanol (v/v), and 0.04% bromphenol blue] in a ratio of 4:1 were boiled for 5 min. Samples (10 μg of protein) were loaded onto 12% gels and subjected to electrophoresis (150 V, 80 min). The separated proteins were transferred to nitrocellulose membranes (1 h, 200 mA per gel; Bio-Rad, Hercules, CA). After transfer, the blots were incubated overnight with 5% nonfat milk in TBS containing 0.04% Tween followed by incubation with 1:1000 dilution of the primary antibody for 2 h. The polyclonal rabbit antibodies, directed against the human HO-1, rat HO-1, or rat HO-2, were from Stressgen Biotechnologies Corp. (Victoria, BC, Canada). The polyclonal rabbit antibody directed against the mouse COX-2 and goat antibody against human COX-1 were from Cayman Chemical (Ann Arbor, MI). After washing with TBS, the blots were incubated for 2 h with secondary antibody (1:5000) conjugated with alkaline phosphatase. Finally, the blots were developed using a premixed solution containing 0.56 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.48 mM nitroblue tetrazolium in buffer (10 mM Tris-HCl, 100 mM NaCl, 59.3 μM MgCl\(_2\), pH 9.5). The blots were scanned, and the optical density of the bands was measured using Scion Image software (Scion Corp., New York, NY).

Heme Oxygenase Activity Assay. HO activity was assayed in homogenates of endothelial cells as previously described (Chernick et al., 1989). Cell homogenates were incubated with 50 μM heme, 2 mg/ml rat liver cytosol (as a source of biliverdin reductase), 1 mM MgCl\(_2\), 3 units of glucose-6-phosphate dehydrogenase, 1 mM glucose 6-phosphate, and 2 mM NAD\(^+\) in 0.5 ml of 0.1 M potassium phosphate buffer, pH 7.4, for 30 min at 37°C. The reaction was terminated by placing the tubes on ice and bilirubin was extracted with chloroform (Chernick et al., 1989). The amount of bilirubin generated was determined using a dual beam scanning spectrophotometer (Lambda 17 UV-visible; PerkinElmer Instruments, Norwalk, CT) and is defined as the difference between 464 and 530 nm (extinction coefficient: 40 mM\(^{-1}\) cm\(^{-1}\) for bilirubin). Results were expressed as nanomoles of bilirubin/milligram of protein/hour.

Cellular Heme Content. Endothelial cells were washed twice with cold 0.15 M KCl and harvested by scraping the flashes with a rubber policeman. Cells were harvested in an Eppendorf tube, and heme content was measured by the pyridine hemochromogen method (Fuhrhop and Smith, 1975). Briefly, cells were resuspended in 0.9% NaCl and mixed with a solution of 25% (v/v) pyridine in 0.075 M NaOH, and the heme content was measured by using the reduced solution minus the oxidized difference spectrum between 390 and 600 nm. The absorbance peak corresponding to the heme band at 418 nm was measured at 37°C. The data are presented as mean ± S.E. for the number of experiments. Statistical significance (p < 0.05) was
determined by the Fisher method of multiple comparisons. For comparison between treatment groups, the null hypothesis was tested by single-factor analysis of variance for multiple groups or unpaired t test for two groups.

**Results**

**Modulation of HO Activity in RCME and RCME-HHO-1 Cells.** As seen in Fig. 1A, RCME cells treated with CoCl2 (150 μM) or heme (10 μM) resulted in an increase of HO-1 mRNA without a significant increase in HO-2 or GAPDH mRNA. Similarly, RCME cells transfected with human HO-1 gene (RCME-HHO1) expressed the human HO-1 mRNA, but there was no increase in human HO-1 gene expression following treatment with CoCl2 or heme due to the absence of promoter in the transduced gene (Fig. 1B). However, rabbit HO-1 in cells transfected with the human HO-1 gene responded to the HO inducers, CoCl2 and heme, in a manner similar to that of nontransfected cells (Fig. 1, B compared with A) without a significant modulation of GAPDH (Fig. 1B) or HO-2 expression (data not shown).

Western blot analysis demonstrated that nontransfected RCME cells contain low basal levels of HO-1 protein (Fig. 2A). The addition of heme or CoCl2 markedly increased the protein levels of the HO-1 isoform. Nontransfected RCME cells expressed HO-2 protein, the levels of which were not significantly altered following treatment with either heme or CoCl2 (Fig. 2A). Figure 2B contrasts the effect of heme on HO protein levels in nontransfected cells and cells transfected with the human HO-1 cDNA. Immunoblot using antibodies raised against human HO-1 protein indicated the presence of the human HO-1 protein in transfected RCME-HHO-1 cells and the lack of effect of heme on its levels (Fig. 2B). Immunoblot with antibodies against rat HO-1, which cross-react with rabbit HO-1, indicated the presence of low levels of HO-1 in both cell types and its induction following exposure to heme in both RCME and RCME-HHO-1 cells (Fig. 2B). It also showed that levels of HO-2 were similar in both cell types and that they are not significantly increased following incubation with exogenous heme (Fig. 2B).

The increase in HO expression brought about by HO-1 gene transfer was accompanied by augmentation in HO activity (Fig. 3A). Importantly, cellular heme content in RCME-HHO-1 cells was 56% lower than that in RCME cells (0.21 ± 0.02 versus 0.48 ± 0.03 nmol of heme/mg of protein), indicating that chronic augmentation in HO activity in cells expressing human HO-1 cDNA brings about depletion of cellular heme levels. As shown in Fig. 3B, HO activity in RCME cells was greatly increased by exposure to CoCl2 for 24 h, reaching levels similar to those detected in RCME-HHO-1 cells. Treatment with heme also increased HO activity, but the effect was less prominent than that of CoCl2. Treatment with SnMP (10 μM), an inhibitor of HO activity, for 24 h inhibited basal level by 80% (from 1.18 ± 0.11 to 0.24 ± 0.05 nmol of bilirubin formed/mg of protein/h; n = 3; p < 0.05), heme-stimulated activity by 70% (from 1.52 ± 0.17 to 0.46 ± 0.07 nmol of bilirubin formed/mg of protein/h; n = 3; p < 0.05), and CoCl2-stimulated levels by 68% (from 4.01 ± 0.37 to 1.29 ± 0.05 nmol of bilirubin formed/mg of protein/h; n = 3; p < 0.05).

**Effect of HO Induction and Inhibition on Prostaglandin Levels in RCME Cells.** Under control conditions, the levels of PGE2 in the culture medium of RCME cells were approximately 20 times higher than those of 6-keto-PGF1α, in agreement with previous reports of prostanooid synthesis in
cultured endothelial cells derived from microvessels (Gerritsen et al., 1988). The addition of heme (10 μM) to the culture medium increased the levels of 6-keto-PGF\textsubscript{1α} and PGE\textsubscript{2} by 62 and 50%, respectively (Fig. 4), which is indicative of enhanced prostaglandin synthesis. Interestingly, SnMP (10 μM) did not significantly affect basal or heme-stimulated prostaglandin levels in the culture medium. Hence, PGE\textsubscript{2} levels were 30,130 ± 2,152 and 33,241 ± 2,890 pg/ml in the culture medium of untreated cells and cells treated with SnMP (n = 6, p = 0.50), respectively. Similarly, 6-keto-PGF\textsubscript{1α} levels in untreated cells and cells treated with SnMP were 1,645 ± 103 and 1,845 ± 103 pg/ml, respectively (n = 6, p = 0.20). Moreover, in the cells treated with heme (n = 6) and heme + SnMP (n = 3), PGE\textsubscript{2} levels were 44,620 ± 1,656 and 42,370 ± 2,627 pg/ml, respectively, whereas 6-keto-PGF\textsubscript{1α} levels amounted to 2,799 ± 402 and 2,222 ± 131 pg/ml in heme- and heme + SnMP-treated cells, respectively.

Treatment of RCME cells with CoCl\textsubscript{2} (150 μM) for 24 h, which potently induced HO-1 expression and HO activity in these cells, greatly decreased the levels of 6-keto-PGF\textsubscript{1α} and PGE\textsubscript{2} in the culture medium (Fig. 4). In another set of experiments, RCME cells were pretreated with CoCl\textsubscript{2} for 24 h, after which CoCl\textsubscript{2} was withdrawn from the culture by washing the cells with phosphate-buffered saline and adding fresh medium for an additional 24 h. CoCl\textsubscript{2} withdrawal resulted in a return to normal levels of HO activity, PGE\textsubscript{2}, and 6-keto-PGF\textsubscript{1α} (data not shown).

Prostaglandin Levels and COX Expression in RCME Cells Expressing Human HO-1. The basal levels of 6-keto-PGF\textsubscript{1α} and PGE\textsubscript{2} in culture medium were decreased by 85% in cells expressing the human HO-1 (RCME-HHO-1 cells) compared with untransduced RCME cells (Fig. 5). RCME cells transduced with the empty expression vector expressed a level of PGE\textsubscript{2} and 6-keto-PGF\textsubscript{1α} similar to that seen in untransduced cells (data not shown). Additional experiments in RCME-HHO-1 cells revealed that incubation of cells with heme increased the levels of PGE\textsubscript{2} by 2-fold (from 4016 ± 717 to 8264 ± 758 pg/ml; n = 3, p < 0.001). The increase in the levels of 6-keto-PGF\textsubscript{1α} in the culture medium of RCME-HHO-1 following heme was not significant (353 ± 96 and 426 ± 15 pg/ml in untreated RCME-HHO-1 and cells treated with heme, respectively).

The effect of overexpressing HO-1 on protein levels of COX isoforms was examined. As shown in Fig. 6, the protein levels of COX-1, the constitutive isoform, were higher in RCME-HHO-1 compared with nontransfected RCME cells. Moreover, addition of heme slightly increased the levels of COX-1 in RCME cells and in RCME-HHO-1. In contrast, COX-2 protein levels were significantly lower in RCME-HHO-1 cells compared with control RCME cells. Like COX-1, COX-2 protein increased in response to heme treatment (Fig. 6).

Effect of Heme on Prostaglandin Levels and COX Expression in Primary Cultures of Endothelial Cells. High-passage cultured endothelial cells may differ function-
ally from first-passage cultured endothelial cells, which are less likely to dedifferentiate. Therefore, additional experiments using primary cultures of BAEC were performed to assess the effect of the heme-HO system on prostaglandin levels and COX expression. As shown in Fig. 7A, incubation of cells with heme for 24 h increased 6-keto-PGF$_{1\alpha}$ and PGE$_2$ by 2- and 4-fold, respectively (Fig. 7A). Western blot analysis showed that incubation of primary cultures with heme resulted in up-regulation of HO-1 with no significant effect on either HO-2, COX-1, or COX-2 protein levels (Fig. 7B). The lack of effect of heme on COX protein may be due to the presence of heme levels in primary cells as compared with cell lines.

**Discussion**

This study demonstrates, for the first time, that the heme-HO system of endothelial cells participates in the regulation of prostaglandin production by these cells, presumably by influencing the availability of heme for the manufacture of catalytically active COX. Two key findings substantiate this conclusion.

The first key finding is that the production of the COX products PGE$_2$ and 6-keto-PGF$_{1\alpha}$ (the nonenzymatic derivative of PGF$_2\alpha$) was greatly diminished in RCME cells treated with CoCl$_2$ or transfected with the human HO-1 gene. In agreement with previous reports (Schwartzman et al., 1986), treatment with CoCl$_2$ increased the expression of HO-1 in RCME cells, enhancing HO activity and presumably decreasing cellular heme, as shown elsewhere (Abraham et al., 1995). Transfection of RCME cells with the human HO-1 gene resulted in augmentation of HO-1 expression, elevation of HO activity, and reduction of cellular heme levels. Hence, the production of COX products by vascular endothelial cells appears to be down-regulated in experimental settings in which HO-1 is overexpressed and cellular heme is reduced.

The second key finding is that treatment of cultured endothelial cells, RCME or BAEC, with heme increases the production of PGE$_2$ and 6-keto-PGF$_{1\alpha}$. In agreement with previous reports, this intervention also caused induction of HO-1 with attendant augmentation of HO activity in endothelial cells (Abraham et al., 1995). The activation of heme-degrading mechanisms in endothelial cells exposed to exogenous heme is regarded as a homeostatic response to augmentation of cellular heme levels (Deramaudt et al., 1998; Wagener et al., 1999). In our study, the stimulatory effect of heme on prostaglandin production by RCME cells was not altered by concurrent treatment with the HO inhibitor SnMP. Accordingly, up-regulation of prostaglandin production in endothelial cells treated with heme may be ascribed to heme itself rather than to a product of its metabolism by HO.

A priori, up-regulation of prostaglandin production by heme in endothelial cells may result from an increase in the amount of arachidonic acid that is available to COX as well as an increase in the expression and/or activity of COX. There is no evidence, however, that cellular heme influences either the rate of arachidonic acid acylation or reacylation, the balance of which determines the amount of arachidonic acid available for prostaglandin synthesis (Parooqui et al., 2000). On the other hand, it is known that heme bound to histidine residues of the peroxidase binding site of COX isoforms is required for catalytic activity (Smith and Marnett, 1991). Therefore, it is likely that the alterations in prostaglandin production observed in endothelial cells subjected to interventions that increase or decrease cellular heme are the result of directional alterations in the levels of catalytically active COX.

According to our study, the expression of COX-1 protein in RCME cells transfected with the human HO-1 gene does not correlate well with the production of prostaglandins. We found that although human HO-1-expressing cells contain more COX-1 protein than nontransfected cells, they produce less prostaglandins. One interpretation of these observations is that COX-1 protein manufactured by endothelial cells expressing human HO-1, although up-regulated, is compromised in terms of catalytic activity because of a less than optimal heme availability. Another possibility is that prostaglandin production in RCME cells is driven primarily by COX-2, the expression of which is significantly down-regu-

![Fig. 5. Effect of HO-1 overexpression on PGE$_2$ and 6-keto-PGF$_{1\alpha}$ levels. The levels of 6-keto-PGF$_{1\alpha}$ and PGE$_2$ in the culture medium of RCME and RCME-HHO-1 cells were measured by EIA as described under Materials and Methods. The results are expressed in picogram of prostaglandins in 1 ml of culture medium and are the mean ± S.E. (n = 3; *, p < 0.05 compared with the corresponding control).](image1)

![Fig. 6. Effect of transfection of RCME with HHO-1 on COX proteins. Western blot analyses for COX-1 and COX-2 were performed on 10 μg of protein isolated from RCME and RCME-HHO-1. These cells were incubated with heme (10 μM) for 24 h. This blot is representative of Western blot analyses performed in six separate experiments.](image2)
lated in cells transfected with the human HO-1. However, preliminary experiments using a selective COX-2 inhibitor (NS398) suggested that COX-2 is not the primary source for the generation of PGE2 and PGI2 in these cells.

The design of the current study did not permit examination of additional mechanisms by which alterations in status of the heme-HO system of endothelial cells impact on prostaglandin production. For example, it is conceivable that reductions in cellular heme decrease PGI2 synthase expression, since this enzyme, like COX, is a heme protein (Tanabe and Ullrich, 1995). Consideration also should be given to the possibility that CO formed during metabolism of heme by HO influences the enzymatic activity of PGI2 synthase (Tanabe and Ullrich, 1995). Our results demonstrating that, in RCME cells expressing human HO-1, addition of heme greatly increased PGE2 levels without affecting the levels of 6-keto-PGF1α support these assumptions.

In summary, the present study documents a regulatory action of the heme-HO system in endothelial cells on prostaglandin production. Up-regulation of HO-1 leading to reduction in cellular heme brings about a decrease of prostaglandin synthesis. We take this finding as indicative that variations in cellular heme levels impact prostaglandin production in endothelial cells by influencing the amount of catalytically active COX.

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References


**Address correspondence to:** Dr. Nader G. Abraham, Professor and Director of Gene Therapy, Department of Pharmacology, New York Medical College, Valhalla, NY, 10595. E-mail: nader_abraham@nymc.edu