Hypoxia activates heparanase expression in an NF- κ B dependent manner

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Abstract. Hypoxia was shown to increase tumor cell invasion into the extracellular matrix *in vitro*. This result suggests that heparanase (Hpa), one of the key enzymes involved in tumor invasion and metastasis, may be regulated by hypoxia. RT-PCR, Western blot and Matrigel invasive assays were used to study the regulation of Hpa under hypoxia in human pancreatic MIA PaCa-2 cancer cells. Compared with those in normoxia (20% O₂), Hpa mRNA, protein and enzymatic activity levels, were up-regulated by a reduction in the oxygen level (1% O₂). Invasion by tumor cells into the extracellular matrix was found to be significantly enhanced. A reduction in Hpa protein levels was observed when nuclear factor κ B (NF- κ B) activation was blocked by pyrrolidine dithiocarbamate. The levels of Hpa were also reduced when Hpa was inhibited by an Hpa-specific antisense oligonucleotide. The MMP-9 mRNA, protein and gelatinase B activity levels in supernatants decreased significantly when Hpa was inhibited. We conclude that up-regulation of Hpa by hypoxia is NF- κ B-dependent in MIA PaCa-2 cells and inhibition of Hpa reduces MMP-9 activity. This reduction in MMP-9 activity may be an important mechanism in tumor metastasis.

Introduction

Heparanase (Hpa) is an endo- β -glucuronidase that cleaves the heparin sulfate (HS) side-chain. This enzymatic reaction not only enhances tumor cell invasion and migration, but also releases important HS-binding cytokines relevant to angio-

genesis, wound healing and tumor growth. Up-regulation of Hpa has been demonstrated in a number of primary human cancers and correlates with reduced postoperative survival in bladder (1), stomach (2), pancreas (3), colorectal (4) and gallbladder cancers (5). In addition, high levels of Hpa are expressed in patients with lymph nodes and distant metastasis (4-7).

In solid tumors, cells are constantly exposed to chronic or acute hypoxia (8). *In vitro* studies have reported that tumor cells exposed to hypoxia exhibit an increased ability to invade the extracellular matrix (ECM) (9,10). Consequently, understanding the regulation and mutual relationship of degrading enzymes in hypoxic conditions is particularly important. It has been shown that high level of heparanase molecules may serve some species to adapt to severe hypoxic environment in *spalax* blind subterranean mole rat (11). In addition, a recent study showed that heparanase gene expression was increased in the brain tissue of rats subjected to repeated hypoxic exposures (12). Hpa activity was shown to be up-regulated by hypoxia in ovarian OC-MZ-6 cancer cell (13). However, information describing the mechanism of the regulation of Hpa and the relationship between degrading enzymes and hypoxia remains unresolved.

In this study, we have characterized the regulation of Hpa in hypoxia using the human pancreatic cancer cell line MIA PaCa-2. The results showed that Hpa mRNA, protein and enzymatic activity levels were up-regulated by hypoxia, which is closely related to enhanced tumor cell invasion. Furthermore, up-regulation of Hpa expression by hypoxia was partly controlled in a nuclear transcriptional factor κ B (NF- κ B)-dependent manner. Inhibition of Hpa reduced metalloproteinase-9 (MMP-9) expression and activity.

Materials and methods

Cell culture. The pancreatic MIA PaCa-2 cancer cells were purchased from the American Type Culture Collection (ATCC) and grown in Dulbecco's minimal essential medium (DMEM; Invitrogen-Gibco, CA) supplemented with high-glucose (4.5 g/l), 10% fetal calf serum (FCS), 100 units/ml penicillin and 100 mg/ml streptomycin. Hypoxia conditions

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Table I. Primers for PCR and their parameters.

Genes	Sense and antisense primer	Product length (bp)	Cycles
Heparanase	5'-tgctactccgagaacactac-3' (exon-3) 5'-atacttgctcatcaccact-3' (exon-9)	705	30
MMP-2	5'-tgacggtaaggacggactc-3' (exon-3) 5'-tggaagcggatggaac-3' (exon-5)	324	35
MMP-9	5'-tcctgggagacctgAgaacc-3' (exon-1) 5'-cggcaagtcttcgagtagtt-3' (exon-3)	308	35
GAPDH	5'-tcctgcaccaccaactgctt-3' (exon-6) 5'-tccaccacctgttgctgta-3' (exon-8)	527	30

were created as previously described (14,15). Cells were cultured in humidified air at 37°C and incubated in either hypoxia (1% O₂, 5% CO₂ and 94% N₂) or normoxia (20% O₂, 5% CO₂ and 75% N₂) conditions.

Transfection. The phosphorothioated antisense oligodeoxynucleotide (AS-ODN) sequence 5'-GGC TTC GAG CGC AGC AGC AT-3' (16) that specifically targets the Hpa mRNA (GeneBank Accession No. AF 155510) in the translation initiation region was synthesized and transfected into the MIA PaCa-2 cells. The non-sense oligodeoxynucleotide (NS-ODN) 5'-TCA GCT AGC GAG GCT GCG CA-3' sequence, containing the same base pairs as the AS-ODN but in a random arrangement, was used as the control.

Cells were cultured in 25-cm² flasks until subconfluence, harvested after treatment with a 0.5% trypsin/0.02% EDTA solution, and seeded in 6-well plates with medium at a concentration of 3×10⁵ cells/well. The medium was changed from the high-glucose DMEM (4.5 g/l) to a low-glucose DMEM (1.5 g/l) supplemented with 1% FCS and antibiotics when the cells reached 60-70% confluence. After 24 h, the transfection was initiated by exchanging the low-glucose DMEM with serum-free medium. Lipofectamine (Invitrogen) and AS-ODN were prepared, mixed and incubated for 20-30 min at room temperature according to the manufacturer's procedure. The solution consisting of the oligodeoxynucleotide/lipofectamine complex was directly added to the wells. After incubation for 8 h, the supernatants were removed, replaced with fresh low-glucose DMEM (1.5 g/l) supplemented with 1% FCS, and the cells were incubated at 37°C under normoxic or hypoxic conditions.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from the MIA PaCa-2 cells with Trizol reagent (Invitrogen) and quantified by UV absorption. Reverse transcription of the RNA was performed using MMLV reverse transcriptase (MBI Fermentas, Lietua) and oligo(dT) primers. The resulting single-stranded cDNA was amplified using a 2X TaqDNA PCR Master Mix (Tiangen, Ltd., Beijing, China). The PCR parameters for Hpa, MMP-2, MMP-9 and GAPDH are outlined in Table I. PCR products were separated by 1.0% agarose gel electrophoresis and

visualized by ethidium bromide staining and quantified by a complete gel documentation and analysis system. The mRNA expression levels were determined by taking the mRNA ratio between the target and the GAPDH genes.

Preparation of the cytoplasm and nuclear proteins and Western blotting. Cytoplasm and nuclear proteins were extracted from the cells cultured in normoxic or hypoxic conditions as previously described (17). Equal amounts of 50 µg (for cytoplasm) and 20 µg (for nucleus) proteins were loaded on to a 10% SDS polyacrylamide gel (SDS-PAGE). After electrophoresis, the proteins were transferred on to a nitrocellulose membrane (Milipore, MA) and blocked using 5% non-fat dried milk dissolved in a solution of TBS-T (1X TBS, 0.05% Tween-20) at room temperature for 2 h. The membranes were rinsed three times in TBS-T and incubated with the anti-human monoclonal Hpa antibody HP-3/17 (1:4000; InSight Biopharmaceuticals, Ltd., Rehovot, Israel), HIF-α (1:200; Santa Cruz), MMP-2 (1:200) and MMP-9 (1:200; Boster, Wuhan, China) at 4°C overnight. The membranes were rinsed three times in TBS-T and incubated with 1:5000 dilution of the secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. After extensive washing with TBS-T, protein bands were visualized by the enhanced chemiluminescence system (Pierce, IL) after exposure of the membranes to film for 30-60 sec. Protein levels were determined by calculating the ratios between the target and β-actin proteins.

Heparanase enzyme activity assay. Heparanase activities in cell lysates were assayed by a heparin-degrading enzyme assay kit according to the manufacturer's instructions (Takara Bio, Inc.). The heparanase activities in all samples were interpolated from a standard curve generated using heparin sulfate as a standard substitute. The absorbance at 450 nm was measured with a spectrophotometric plate reader.

Cell invasion assay. Invasion of the cells through a Matrigel assay was determined using 24-well transwell invasion chambers (8.0 µm pore size with polycarbonate membrane; Corstar Scientific, MA) in accordance with the manufacturer's instructions. Briefly, the chambers were coated with Matrigel

Figure 1. Expression of Hpa under normoxic or hypoxic conditions in pancreatic MIA PaCa-2 cancer cell. Semi-quantitative RT-PCR and Western blot results show that both mRNA (A) and protein (B) levels were up-regulated by hypoxia (1% O₂, P<0.05). Nuclear HIF-1α protein expression was also up-regulated in hypoxia (P<0.05). Lanes: M, DNA marker; N, normoxia (20% O₂); B, blank control; 1-5, hypoxia at 3, 6, 12, 24 and 48 h. *P<0.01 vs. normoxia.

(10 μg/well; BD Biosciences, MA) for 4 h and prehydrated with serum-free DMEM for 2 h before use. Cells transferred or non-transferred with AS-ODN were trypsinized and washed twice with serum-free DMEM. The cells were subsequently (1x10⁵/well) suspended in 400 μl serum-free DMEM and seeded in to the top chamber of the transwell insert. Medium (600 μl) that had been conditioned by NIH₃T₃ mouse fibroblasts was used as a chemoattractant and placed in the lower compartment of the chamber. After incubation in normoxia or hypoxia conditions for 24 h at 37°C, cells located on the inner side of the chamber were removed using cotton swabs. Cells that had migrated through the filter insert to the opposing surface were fixed by 4% paraform and stained with hematoxylin and eosin staining. The membrane was sliced and fixed on to slides and the cells were counted under a light microscope.

Zymography. Zymography was used for the analysis of MMP-2 and MMP-9 levels in cultured medium. Cells were cultured, maintained and transfected as above. The medium was subsequently exchanged with 5 ml of fresh serum-free and low-glucose medium and incubation of the cells continued under hypoxic conditions. The medium was collected and concentrated 5-fold at -80°C before analysis. Aliquots of the conditioned medium were separated on a 10% SDS-PAGE with 0.5 mg/ml gelatin (Amersco, Inc., OH) under non-denaturing conditions. After electrophoresis, the gels were incubated in 1% Triton X-100 for 2 h to remove the SDS and subsequently incubated for 42 h in substrate buffer (50 mM Tris-HCl, 5 mM CaCl₂, 1.0 μM ZnCl₂ and 0.02% NaN₃, pH 7.5) at 37°C. Gels were stained in a solution containing 0.1% Coomassie Blue R₂₅₀, 50% methanol and 10% acetic acid for 1 h and destained in a solution of 30% methanol and 10% acetic acid. Bands with gelatinolytic activity were clearly visible. Proteinases were identified on the basis of their molecular size.

Figure 2. Expression of Hpa in MIA PaCa-2 cell with Hpa AS-ODN transfection at 24 h. Compared to normoxia, Hpa level was increased by hypoxia and decreased by Hpa AS-ODN transection in a dose-dependent manner at 100, 200 and 400 nM (P<0.05). No changes were revealed by transfection reagents and NS-ODN in the same concentrations. (A) mRNA expression by RT-PCR; (B) protein expression by Western blotting. M, DNA marker; N, normoxia; H, hypoxia; lane 1, transfection reagents; lanes 2-4, NS-ODN at 100, 200 and 400 nM concentration; lanes 5-7, AS-ODN at 100, 200 and 400 nM concentration.

Electrophoretic mobility shift assay (EMSA) of nuclear NF-κB. Cells were cultured in normoxic or hypoxic conditions and pyrrolidine dithiocarbamate (PDTC), a specific inhibitor of NF-κB, was added to the medium at a concentration of 50 μM. After 6, 12 and 24 h, the cells were collected and the nuclear proteins extracted as described above. NF-κB oligonucleotides used for the gel shift analysis were: 5'-AGT TGA GGG GAC TTT CCC AGG C-3' and 5'-TCA ACT CCC CTG AAA GGG TCC G-3'. Nuclear extracts (20 μg/sample) were incubated with ³²P-labeled oligonucleotides, resolved on non-denaturing 8% polyacrylamide gels and visualized by autoradiograph.

Statistical analysis. Data in figures are presented as mean ± SD of at least three different experiments performed in triplicate. Statistical significance was analyzed using Student's test or one-way analysis of variance. The value of P<0.05 was considered significant.

Results

Hypoxia enhances Hpa expression in pancreatic MIA PaCa-2 cancer cells. As shown in Fig. 1, the level of Hpa expression was relatively low in normoxia (20% O₂); however, after exposure to hypoxia (1% O₂) for 3, 6, 12, 24 and 48 h, the Hpa expression, both mRNA and protein levels, increased steadily and significantly. At 24 h, the Hpa mRNA and protein levels reached its maximum. The nuclear HIF-1α protein expression level was dramatically up-regulated by hypoxia. The results indicated that hypoxia induced an increase in mRNA and protein Hpa levels.

Hpa AS-ODN decreases the Hpa expression level. As shown in Fig. 2, the Hpa expression level had significantly increased following 24 h hypoxia (lane 2) and the presence of Hpa

Figure 3. Hpa activity in the lysates of MIA PaCa-2 cells measured by the Taraka assay. This demonstrates that Hpa activity was increased by hypoxia ($P<0.01$) and decreased by Hpa antisense oligonucleotide, ($P<0.01$), which was consistent with the changes of mRNA and protein level by RT-PCR and Western blot analysis. N, normoxia; H, hypoxia; H+AS-ODN, hypoxia and antisense oligonucleotide; H+NS-ODN, hypoxia and non-sense oligonucleotide.

AS-ODN at 100, 200 and 400 nM inhibited the Hpa expression at both the mRNA and protein levels in a dose-dependent manner (lanes 7-9). In contrast, no influence on the levels of Hpa expression was observed in the presence of the NS-ODN (lanes 4-6) or the transfection reagent only (lane 3).

Heparanase activity is associated with heparanase expression. As shown in Fig. 3, the heparanase activity increased significantly and reached a maximum at 24 h ($P<0.01$). The activity of heparanase showed a significant decrease ($P<0.01$) in the presence of the Hpa antisense oligonucleotide (400 nM). In contrast, the activity did not dramatically decrease ($P>0.05$) in the presence of the non-sense oligonucleotide.

Hypoxia enhances tumor cell invasion, which is inhibited by Hpa AS-ODN. Following 24 h incubation, the cell number that had migrated across the filter of the transwell invasion chamber and fixed on the lower surface of the membrane in normoxia, hypoxia, hypoxia with NS-ODN (400 nM) and AS-ODN (400 nM) transfection was 74.33 ± 8.14 , 163.33 ± 13.32 , 173.67 ± 7.09 and 72.67 ± 7.50 , respectively (Fig. 4). The data showed that hypoxia increased the level of cell invasion. Quantification revealed that the number of invading cells increased by 1.8-fold when cultured in 1% O_2 compared with cell invasion when cultured in 20% O_2 ($P<0.01$). Furthermore, Hpa AS-ODN transfection reduced the number of invading cells under hypoxia by 55% ($P<0.01$). In contrast, the level of invading cells did not change significantly when the cells were transfected with the NS-ODN ($P>0.05$). This finding revealed that hypoxia enhanced tumor cell invasion by up-regulating Hpa expression.

Down-regulation of Hpa expression inhibits MMP-9 expression and activity. As shown in Fig. 5, when cultured in hypoxia at 6, 12 and 24 h, the MMP-9 mRNA and protein levels were steadily up-regulated in MIA PaCa-2 cells. After the inhibition of Hpa expression, the MMP-9 expression level decreased dramatically, especially at 24 h. However, neither hypoxia nor transfection with Hpa AS-ODN affected the MMP-2 expression.

Consistent with the above results, the zymographic analysis also showed some interesting changes (Fig. 5d). Three main gelatinolytic bands corresponding to molecular masses of

Figure 4. Matrigel invasive assay of MIA PaCa-2 cells under normoxic or hypoxic conditions. (A) Cells were seeded onto transwell chambers at the same density (1×10^5 /well) at 37°C . After 24 h incubation, cells on the low surface of the membrane were fixed and stained with haematoxylin and eosin staining. Photographs indicate invading cells were under normoxia (a), hypoxia (b), hypoxia and transfection with NS-ODN (c) or AS-ODN (d) in 400 nM concentration. (B) Invading cells of each five fields in each well were counted randomly under light microscope at $\times 20$ magnification and averaged numerically. Data indicate mean value $\pm$ SE of three separate experiment all carried out in triplicate. [#] $P<0.01$ vs. normoxia (a) and ^{*} $P<0.01$ vs. hypoxia (b).

92 (latent MMP-9), 72 (active MMP-9) and 62 kDa (active MMP-2) were observed. The gelatinase activity was very low in the supernatant under normoxia. This observation may have resulted from the low-glucose (1.5 g/l) and low-nutrient (1% FCS) medium used. After 6 h exposure to hypoxia, the enzymatic activities were significantly inhibited such that the negative bands were barely visible. However, compared with the bands in normoxia, both MMP-2 and MMP-9, especially their active forms, were dramatically elevated at 12 and 24 h hypoxia. The most interesting change was observed when the Hpa level was down-regulated. Here, the enzymatic activities of MMP-9, either latent or active forms, were significantly suppressed; however, no obvious changes were observed in the negative bands degraded by MMP-2. The results revealed that the inhibition of Hpa may reduce the level of MMP-9 expression and enzymatic activity.

Inhibition of NF- κ B decreases the Hpa protein level. NF- κ B can be activated by hypoxia (18,19) and our results have demonstrated that hypoxia increased Hpa expression. However, whether the NF- κ B signaling pathway plays an important role in the regulation of Hpa remains unclear. As shown in Fig. 6, in normoxia, the activation of NF- κ B was relatively low, whereas NF- κ B activity increased dramatically at 6, 12 and 24 h when MIA PaCa-2 cells were exposed to hypoxia. Additionally, the NF- κ B activation was inhibited by PDTC (50 μM). Consistent with the NF- κ B activation, the Hpa protein expression levels also increased due to hypoxia

Figure 5. Analysis of MMP-2 and MMP-9 expression in hypoxia and with Hpa AS-ODN transfection in MIA PaCa-2 cell by semi-quantative RT-PCR, Western blot and zymography analysis. It indicates that neither mRNA nor protein of MMP-2 (A, C) changed significantly, but MMP-9 mRNA and protein (B, C) were up-regulated by hypoxia and inhibited by Hpa AS-ODN at 12 and 24 h ($P < 0.05$). Photography (D) shows that at 12 and 24 h, gelatinase B (MMP-9) activity, both in latent (92 kDa) and active forms (83 kDa), were up-regulated by hypoxia and suppressed by Hpa AS-ODN ($P < 0.05$). Also, the active form (66 kDa) of gelatinase A activity (MMP-2) was up-regulated ($P < 0.05$) but not down-regulated by Hpa AS-ODN. No latent form (72 kDa) of gelatinase A was revealed in this experiment. Both gelatinase A and B activities were inhibited by hypoxia at 6 h whether transfected with AS-ODN or not. Lanes: M, DNA marker; N, normoxia; H, hypoxia; H+A, hypoxia and AS-ODN.

Figure 6. Western blot analysis of Hpa protein under the condition that NF- κ B signal pathway is blocked by PTDC in MIA PaCa-2 cell. Nuclear protein was incubated with [γ - 32 P]-ATP-labeled oligonucleotide of NF- κ B and catalysed by T_4 polynucleotide kinase. After electrophoresis, the gels were enclosed and exposure to X-ray film at -70°C for 24 h. The Hpa protein was analyzed then the NF- κ B blocked or not. Compared with normoxia, hypoxia activated the NF- κ B signal pathway and increased Hpa protein expression at different times ($P < 0.05$). When NF- κ B was blocked by PTDC (50 μM), the Hpa protein expression decreased significantly ($P < 0.05$). (A) Activation of NF- κ B. (B) Hpa protein expression. Pr, probe only; N, normoxia; H, hypoxia; H+P, hypoxia and PTDC.

and remarkably decreased when the NF- κ B signaling pathway was inhibited.

Discussion

Clearly tumor microenvironmental factors, especially hypoxia, enhance the ability of cells to form metastases by

modifying cellular gene expression both transiently and permanently. However, the role of Hpa in hypoxia has remained unresolved.

In our study, the expression of Hpa, both at the mRNA and protein levels, increased significantly when cells were exposed to 1% O_2 . This observation suggests that Hpa expression is strongly regulated by hypoxia and contrasts the results reported by He *et al* (13). In this study, the authors showed that Hpa enzymatic activity was up-regulated by hypoxia but the protein level remained unchanged. This difference may be due to He *et al* detecting Hpa levels in the medium whereas we have detected Hpa levels in cell lysates. Consequently, as the protein is synthesized and subsequently secreted into the medium, the Hpa levels within and outside the cells may be different at particular times. Additionally, though distributed in the stromal cells, Hpa protein expression is predominately found to occur in tumor cells (5-11). Consequently, detecting the Hpa protein levels inside the cell should more accurately reflect the amount of Hpa present in the cell. Finally, small changes in protein content in the medium may be difficult to accurately measure using current methods.

As shown in Fig. 4, the invasion capacity of MIA PaCa-2 cells increased dramatically in hypoxia and was effectively inhibited by Hpa AS-ODN. This observation indicates that hypoxia-inducible Hpa expression is closely related to the invasive behavior of tumor cells. However, the exact mechanism of Hpa up-regulation by hypoxia is not distinct. NF- κ B is an important transcriptional factor which regulates a number of genes involved in immunoreactions, apoptosis, tumor genesis and therapeutic resistance. In hypoxia, NF- κ B can easily be activated *in vitro* in normal and transformed cells (18,19). Research on the relationship between NF- κ B and Hpa activity is limited and conflicting. For example,

Andela *et al* found that mutant I κ B (mI κ B), which contained a dominant negative form of I κ B α and cannot dissociate from NF- κ B in response to stimulation of the I κ B kinase pathway, reduced the Hpa expression in murine alveolar carcinoma cells (20). In contrast, inhibition of NF- κ B did not decrease Hpa expression induced by tumor necrosis factor- α (TNF- α) and interleukin-1 (21). We previously reported that heparanase and NF- κ B proteins up-regulated in pancreatic cancer tissue (22). In this study, consistent with the activation of NF- κ B, Hpa levels showed a dramatic increase by hypoxia. Furthermore, inhibition of NF- κ B activation by PDTC led to a significant decrease in the levels of Hpa. This observation strongly suggests that the NF- κ B signaling pathway plays a pivotal role in the regulation of Hpa by hypoxia.

Both Hpa and MMPs are ECM-degradation enzymes and essential to tumor invasion, but to our knowledge, their relationship in tumor progression has not been explored. As shown in Fig. 5, one of the important findings revealed in this study was the observation that MMP-9 activity in supernatants, both in the inactive and active forms, was noticeably inhibited at 12 and 24 h by Hpa AS-ODN. The possible reason for this observation is that cell adhesion is a prerequisite for cell invasion and some adhesion molecules act as anchors for MMPs localized at the cell-matrix interface. For example, $\alpha_5\beta_3$ integrin enables active MMP-2 to localize to the cell surface and this integrin has been suggested to be involved in modulating MMP-2 activation (23). As Hpa contains a trans-membrane domain, this protein is a membrane-associated enzyme (24). The cell surface-associated Hpa promotes adhesion of otherwise non-adherent lymphoma cells which are independent of Hpa enzymatic properties (25). As an adhesive molecule, Hpa may anchor and activate MMP-9 at the cell surface. The number of Hpa-mediated cell surface anchors for MMP-9 to interact with would be reduced when Hpa expression is down-regulated. This would result in a reduction in the levels of MMP-9 activity. Conversely, Mollinedo *et al* found that Hpa and MMP-9 co-localized in the gelatinase-rich granules, named tertiary granules of human resting neutrophils, and readily mobilized to the cell surface. The treatment with TNF- α or granulocyte-macrophage colony-stimulating factor induced the release of Hpa and MMP-9 at similar percentage levels (26). MMP-9 may be released in a complex with Hpa during cell activation. In situations where Hpa expression is down-regulated, a reduction in the amount of MMP-9 released would lead to the inhibition of gelatinase B activity. However, this concept does not account for the observation that the MMP-9 mRNA levels were also down-regulated by Hpa AS-ODN. As such, while the Hpa protein may act as an 'anchor' or complex with the MMP-9 protein, this potential function of Hpa could not influence the observed change in the MMP-9 mRNA levels. Reports discussing the activity relationship between Hpa and MMP-9 are missing and require further studies.

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