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1．研究テーマ

血管内皮細胞におけるトロンビンによる誘導されたcox-1遺伝子のSp1依存の解

2．本年度の研究業績

(1) 学会・研究会等における発表　(有)・無　(学会名・演題)

XIIth International Vascular Biology Meeting.
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(2) 学会誌等に発表した論文　(有)・無　(雑誌名・論文名)

Transcription Factor Sp1 Is Essential for cox-1 Gene Expression Upregulated By Thrombin (投稿中)

3．今後の研究計画

いままでの研究ではまた解決しなかったことが残っていますので引き続きしたいと思っています。今後も助成金をお願い申しあけます。

4．指導責任者の意見

留学生王傑は外科医であるが、向学心が強く、当研究室において血管新生における Sp 1 のメカニズムに関して基礎的な研究に従事している。人一倍研究熱心で、連日連夜真摯に研究に取り組んでいる。常に一流の国際雑誌に目を通し、関連ある研究論文を集めて勉強し、教室内のセミナーで紹介するなど積極性が見られる。大学院の研究に関してもすでに細胞培養や遺伝子工学の技術、特に RNA, DNA の精製、PCR による遺伝子の増幅、シークエンス解析、ベクターの作製、遺伝子発現の検出法（ノーザンブロット、ウエスタンブロット、ゲルシフト）などを完全に習得し、興味深い成果を挙げつつある。本年、大学院の最高学年に達したこともあって、研究者、指導者としての自覚も多少出てきた。明るい性格で協調性もあるので、下級生の面倒を見させているが、熱心に研究指導にも精を出している。

指導責任者氏名 宮田誠逸

5．研 究 報 告 書

別紙報告書作成要領により、添付の用紙で研究報告書を作成して下さい。

研究発表中または研究中の本人のスナップ写真を添付して下さい。

※研究成果を発表する場合は、発表原稿・抄録集等も添付して下さい。

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—日中医学協会助成事業—

血管内皮細胞におけるトロンビンによる誘導された Cyclooxygenase-1 遺伝子の Sp1 の役割解明

(Transcription Factor Sp1 Is Essential For Cyclooxygenase-1 Gene Expression Upregulated By Thrombin)

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Abstract

Transcriptional factor Sp1 has been implicated in the expression of many genes. Moreover, it has been suggested that Sp1 is linked to the maintenance of methylation-free CpG island, the cell cycle, and the formation of the active chromatin structures. We investigated the effects of thrombin on the expression of the Cyclooxygenase-1. in the Bovine Arterial Endothelial Cell. Treatment with thrombin induced synthesis of PGI2. Thrombin induced a transient increase of Cyclooxygenase-1 mRNA accumulation in BAE cells, without affecting cox-2 mRNA level. The accumulation of cox-1 mRNA peaked at 30 min and decreased to control levels by 3 hours, which was associated with a time-dependent increase of cox-1 protein expression in response to thrombin. Both increase in cox-1 mRNA and protein expression in response to thrombin were inhibited by Hirudin, a thrombin receptor antagonist. In addition, the luciferase assay showed that thrombin induced an about 10-fold increase in the cox-1 promoter activity compared with the control. This enhanced KDR promoter activity was also abolished by Hirudin. The deletion analyses indicated that a sp1-containing region (from -115 to –97 bp) within the cox-1 promoter gene was required for the enhanced cox-1 expression induced by thrombin. Band shift assays confirmed specific binding of Sp1 to the segment. Mutation of either Sp1 binding site significantly reduced the promoter activity. These finding suggest that thrombin could induce cox-1 activity and increase PGE2 synthesis. Binding of Sp1 to Sp1 site on the promoter region activites the cox-1 gene transcription induced by thrombin.

Key words thrombin, PGI2, cox-1 promoter activity, Sp1

Introduction

Prostaglandins are important mediators of a wide varity of physiological processes (1). There are two isozymes, Cox-1 and Cox-2, that initiate prostanglandin synthesis (2-3). Both enzymes catalyze the production of prostaglandins (PGs) from arachidonic acid (AA), generated by phospholipases A2 (PLA2s), a family of acyl esterases that cause the release of AA from cellular phospholipids. Although indistinguishable in their biosynthetic catalytic activities, the two isozymes appear to have different physiological functions. Cox-1 is thought to be a constitutively expressed enzyme (4), and the expression of Cox-2 is inducible by cytokines or other stimulati in a variety of cell types (5).

Cox-1 expression might also be regulated through extracellular signaling mechanism. Although Cox-1 has been described as a constitutive enzyme (whereas synthesis of Cox-2 has been shown to be inducible), it has now clear that Cox-1 can be induced by cytokines under appropriate circumstances. For example c-kit ligand, either alone or in combination with IL-3, IL-9, or IL-10、was able to induce expression of Cox-1 in mouse mast cells (6). Similarly, stem cell factor, especially when combined with dexamethasone, can induce Cox-1 expression in these cells (7). A recent study also demonstrated induction of Cox-1 synthesis in cells following treatment with basic FGF, although with a prolonged time course compared to induction of Cox-2 expression (8).

Cox-2-overexpression cells produce prostaglandins, proangiogenic factor, and stimulate both endothelial migration and tube formation, while control cells have little activity. The effect is inhibited by antibodies to combinations of angiogenic factor, by NS-398 (a selective Cox-2 inhibitor), and by aspirin. NS-398 does not inhibit production of angiogenic factor or angiogenesis induced by Cox-2-negative cells. Treatment of endothelial cells with aspirin or a Cox-1 antisense oligonucleotide inhibits Cox-1 activity/expression and suppresses tube formation. Cyclooxygenase regulates colon carcinoma-induced angiogenesis by two mechanisms: Cox-2 can modulate production of angiogenic factor by colon cancer cells, while Cox-1 regulates angiogenesis in endothelial cells (9).

Cox-1 overexpressing expressed high levels of functional Cox-1 polypeptide in the endoplasmic reticulum and the nucleus. In vitro proliferation of these cells was reduced compared with vector-transfected ECV cells. Cox-1 overexpression also enhanced the tumor necrosis factor-a-induced apotosis of ECV cells 2-fold. Incontrast to in vitro behavior, ECV cells proliferated aggressively and formed tumors in athymic "nude" mice, the growth of Cox-1-induced tumors was inhibited by indomathacin, suggesting that Cox-1 overexpression in immortalized ECV endothelial cells results in nuclear localization of the polypeptide and tumorigensis (10).

Mice homozygous for a disrupted Cox-1 gene had no gross or microscopic pathological changes in the gastrointestinal tract, and showed less gastric ulceration than wild-type mice after gavage with indomethacin(11). Similarly, mice deficient in Cox-2 also showed no spontaneous gastric ulceration or accompanying histopathological abnormalities of the intestinal epithelium (12). These studies demonsreate that neither Cox-1 or Cox-2 are ssiential for the maintenance of gastrointestinal integrity in basal unstressed condition.

Thrombin is generated from prothrombin in places with injury or inflammation. Thrombin exhibits its effects through activation of the G protein-coupled receptor PAR-1 that belongs to a new family of protease activated receptor (13). Binding to its receptor results in intracellular signal transduction, including the G protein-stimulated phosphatidylinositol metabolism via phospholipase C-β (PLC-β) (14), protein kinase C (PKC) activation (15-16), intracellular calcium (Ca^{2+}) mobilization, and calcium-dependent protein kinase activation (15). Intracellular protein tyrosine kinases are also activated, thereafter leading to the tyrosine phosphorylation. Downstream components of thrombin-activated signaling cascades include Raf-1 and MAP kinase (17). These signaling events lead to nuclear protooncogene transcription activation (c-fos, c-jun, c-myc) (18) and increased expression of endogenous mitogenic factors, which subsequently cause cell proliferation and migration. The recent study had indicted that thrombin induced the expression of Cox-1 (19), however, the mechanism (s) of transcriptional regulation of the Cox-1 gene expression is as yet clearly understood. In this study, we attempt to identify which thrombin regulates Cox-1 gene expression and specific transcriptional factor that play a critical role in the regulation of thrombin-mediated transcriptional activation of the Cox-1 gene.

Material and Method

Cell culture

Bovine arterial endothelial cells (BAE cells) were isolated from bovine carotid artery and maintained as previously described (20). The cells were seeded in 100-mm culture dishes with MEM Medium (Life Technologies Grand Island, NY) containing 10% fetal bovine serum (FBS, JRH Biosciences, Lenexia, KS) and subcultured with 5% FBS-MEM. At confluence, BAE cells were harvested with 0.05% trypin and 0.05% EDTA in PBS and resuspended in 5% FBS-MEM for further experiments. All cells were incultured at 37℃ in 5% $CO_{2,}$ and 95% air. The medium were changed every 2~3 days. BAE cells between passages 10~20 were used for this study.

PGI2 production by cells

BAE cells (1×10^4) were plated in six-well dishs and grown to 60% confluence in growth medium. The cells were then treated as described below. Levels of 6-keta PGI2 released by the cells were measured by enzyme immunoassay. Amounts of 6-keta PGI2 production were normalized to protein concentration.

Extraction of total cellular RNA and RT-PCR of Cox-1 and Cox-2

When cells reached subconfluency, they were washed once with PBS and cultured in the absence or presence of 5U/ml of thrombin for indicated times. For the isolation of RNA, cells were washed once with PBS, and RNA was isolated by using the ISOGENE reagent (clontic) according to the protocol. RT-PCR was performed with the SuperScript One-Step RT-PCR system (GIBCO-BRL) according to the manufacture's instructions. To detect the Cox-1 and Cox-2, the following primers were used as previously reported: sense primer 5'-CAGCGGCTTTTGTGGAAGACTCAC-3' and antisense primer 5'-ACTTCTCGGTGTCACTTCTTGGAC-3' for flt-1, and sense primer 5'-CAACAAAGTCGGGAGAGGAG-3' and antisense primer 5'-ATGACGATGGACAAGTAGCC-3' for Cox-1 (21). As an internal control, mRNA of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was amplified by using the sense primer 5'-CCACCCATGGCAAATTCCATGGCA-3' and the antisense primer 5'-TCTAGACGGCAGGTCAGGTCCACC-3' (21). PCR was performed with 35 cycles of the following amplification protocol: 94°C for 1 minute, 48°C for 1.5 minutes, and 72°C for 1 minute. Ten μl of the amplified PCR products were electrophoresed on a 1% agarose gel (Sigma), stained with ethidium bromide and photographed with a Polaroid camera under UV light.

Immunofluorescence staining of Cox-1 and image analysis

BAE cells (1×10^4) were seeded into 24-well culture plates, thrombin was added at the indicated days in 0.5% FBS-MEM and cultured for 2 days. Cells were washed twice with PBS, fixed with 1.9% formaldehyde, and rinsed three times with PBS. After blocking with PBS containing 0.2% bovine serum albumin (blocking buffer), the cells were incubated with affinity-purified anti- Cox-1 and Cox-2 antibody (Santa Cruz, CA) (1:20 diluted in blocking solution) for 45 min at room temperature and then with the FITC-conjugated goat antirabbit IgG (Sigma) (1:40 solution) for 45 min. The fluorescence image analysis was performed using an interactive laser cytometer ACAS-570 (Meridian, Okemos, MI) and the fluorescence intensities of individual cells were measured. For each condition, three wells were prepared and four areas per well were assayed.

Construction of the Cox-1 5'-flanking region,
5'-deletion and site-directed mutants.

A fragment of the Cox-1 promoter –1261 to +28 designated as wild-type promoter was obstained by PCR using genomic DNA (100 ng) isolated from human planceta (Clontic). It was digested by restriction degiestion by using 5'-Blg II (Promega Corp) position –1261 and 3'-HindIII (Promega Corp) at position +28 and cloned into the pGL3 Bisic plasmid, which was termed as Cox-1 –1261 to +28 according to the length of the fragment from the transcription start site in the 5'- and 3'- direction. Primers were synthesized according to the sequence relative to the ATG codon (A as +1): the upstream primers, -1261 ~ 1237, -916 ~ -899, -744 ~ -728, -565 ~ -547, -257 ~ -240; the downstream primers, +2~+28 Cox-1 –916 ~ +28, -744 ~ +28, -565 ~ +28, and -255 ~ +28 were created by PCR, Cox-1 –1261 ~ +28 was used as a template. The PCR productions were purified from agarose gel, digested by restriction degiestion by using 5'-Blg II and 3'-HindIII (Promega Corp), and cloned into a promoterless luciferase expression vector, pGL3 basic plasmid. To generate the site-directed mutants where the two Sp1 sites were changed, a PCR-mediated site-directed mutagenesis was employed as described previously (22). All constructs were sequenced from the 5' and 3' end to confirm orientation and sequenced by sequence comparison to previously published deta(23).

Transfection and Luciferase Assay

Transient transfections were performed with FuGENE 6 transfection reagent (Boehringer) according to the manufacture's instructions. 1×10^4 Cells were seeded in 24-well tissue-culture plates one day prior to transfection. The pSVβ-Gal and pGL3 were co-transfected as negative control. Cells were incubated in transfection mixture (containing 0.18μg of the appropriate reporter constructs, 0.02μg pSVβ-Gal to correct the variability in transfection efficiency, and 0.4μl FuGENE 6 transfection reagent in a total volume of 1ml MEM per well) for 48h. Luciferase assays were performed using a Luciferase Assay System, and determined with the AML3000 microtiter plate luminometer. The transfection efficiency was normzlized to the β-Galactosidase production by the AURORA™ GAL-XE system (Chemiluminescent Receptor Assay for β-Galactosidase, ICN Pharmaceuticals, Inc. Costa Mesa, CA) according to the protocol.

Electrophoretic Mobility Shift Assay

The shift assay was performed by a previously described procedure (19). A double-stranded oligonucleotide used as Sp1 probe was composed of the sequence 5'-TTCAGGGGGCGGAGCCA-3' and 5'-TTCAGTTTGCGGAGCCA-3' (a mutant with mutanted bases underlined), both synthesized in a Pharmacia oligonucleotide synthesizer (Piscateway, NY). The end-labeling was performed by using Klenow polymerase with [32P] dCTP. The probe contained a consensus Sp1 binding cite and Sp1 mutant in the context of the human Cox-1 promoter from nucleotides –614 ~ 597 relative to the major transcription start site. Nuclear proteins were extracted with Nuclear protein extract kit according to the protocol. The binding reaction mixture contained 0.5 ng of double-stranded oligonucleotide probe, 2 μg of poly (dI).poly (dC), and 5 μg of protein in buffer C, supplemented with 35mM Mg2. After a 20 min incubation at 20°C, the mixture was electrophoresed through a 6% (w/v) polyacrylamide gel in 0.25×Tris borate-EDTA running buffer for 2h. The gel was then dried and subjected to autoradiography. In competition experiments, nuclear extracts or purified Sp1 were incubated for 5 min with unlabeled oligonucleotide or DNA fragment in a 50-fold molar excess perior to the addition of the labeling probe. Super-shift assays were carried out by adding 2 μg of rabbit polyclonal anti-Sp1 body to the DNA/protein mixture for 30 min at 4°C. The band was analyzed on gel electrophoresis as described above. An unrelated rabbit polyclonal anti-Cox-1 antibody was included as negative control.

Statistical analysis

All experiments were performed three times in triplicate. All values are expressed as mean±SE, unless otherwise indicated. Comparison of the effects of different inhibitors, and time points were performed by ANOVA. Multigroup comparisons were carried out using Bonferroni-modified *t*-test. $P < 0.05$ is considered to be statistically significant.

Results

Cox-1 mRNA expression

The effects of thrombin on Cox-1 mRNA are shown in Figure 1. The cells were treated with control medium or medium with thrombin for difference time. Single PCR products were obtained for Cox-1 mRNA at the expected size of 355bp. The effect was evident by 30min, and persisted for at least 24h. Thrombin had no effect on Cox-2 mRNA abundance at any time point.

Cox-1 protein expression

The effects of thrombin on Cox-1 protein expression are shown in Figure 2. We measured the KDR protein expression using a immunofluorescence staining assay. The data in Fig. 2 showed a similar increase in the level of Cox-1 proteins as that of the Cox-1 mRNA, and it is quite clear that thrombin caused an enhancement in the expression of Cox-1 proteins.

Next, to understand whether the thrombin-induced increase in the Cox-1 mRNA and proteins was mediated through transcriptional regulation or not, we examined the effect of thrombin on the Cox-1 promoter activity using a reporter gene assay. A smallest construct containing the full Cox-1 promoter activity (-996 to +68) was inserted upstream of a luciferase reporter gene was transfected into BAE cells, and the luciferase activities were evaluated. As shown in Figure 3, Thrombin increased the Cox-1 promoter activity, which changed in a time- and dose-dependent manner. These data indicate that thrombin enhanced up-regulation of the Cox-1 promoter activity.

Deletion analysis of the human Cox-1 promoter

To identify the essential regions for the promoter activity increased by thrombin, we generated various mutants, in which the 5'-end from -1261 ~ -255 was gradually deleted, while the common 3'-end from –225 to +28was kept unchanged. Then they were inserted into the luciferase reporter plasmid pGL3 and transfected into BAE cells together with pSVβ-Gal (to correct the differences derived from transfection efficiency). The luciferase activity was normalized to that of the pGL3 Control vector. As shown in Fig. 4, the promoter region from –1261 to +28 showed a 10-fold induction of the luciferase activity upon thrombin stimulation as compared with the untreated cells. Deletion of the region from –1261 to –565 did not affect both the basal and the thrombin induced transcriptional activity of the Cox-1 promoter. However, when the construct was further deleted to –255, an about 80% reduction in the transcriptional activity was observed,

and the basal promoter activity was also reduced. This suggests that the region from –565 to –255 is required for the full response of the Cox-1 promoter gene to thrombin; what's more, it is important for the basal transcription of the Cox-1gene.

Analysis by band shift assays of Sp1 binding to the Cox-1 gene

Binding of BAECs nuclear proteins to this region was investigated. Fig 4 shows band shift when nuclear extracts of BAECs were incubated with –565/-255 probe containing the distal action region. Two bands were noted with this probe. Since this region comprise putative binding sites for Sp1 and AP2, a 150-fold molar excess of consensus oligonucleotide containing Sp1 and AP2 sequence was preincubated with nuclear extracts perior to the addition of the probe. Both bands were specifically competed by Sp1 or unlabeled probes but not by AP2 oligonucleotide. Furthermore, a 50-fold molar excess of –565/-255 in which the canonical Sp1 site was mutated failed to inhibit the formation of the two DNA-protein complexes. The band was supershifted with antibody directed against Sp1 but not with unrelated antibody such as anti-Cox-1 antibody. These results indicate that Sp1 closely related proteins bind to the Sp1 site. To further explore he ability of the Sp1 binding site to function as a thrombin response element, we examined whether it could confer thrombin inducibility to a heterologous, minimal pGl3 vector that lacks enhance sequences. An enhanceless pGL3 vector was unresponsive to thrombin. However, when three copies of the Sp1 binding site were inserted into the promoter, thrombin induced promoter activity by 4-fold (p <0.05). Furthermore, insertion of mutated Sp1 sites failed to confer thrombin responsiveness, and base-line promoter activity also was increased by insertion of both Sp1 and mutated Sp1 binding sites. These results indicate that the addition of Sp1 binding sites into a heterologous promoter can convert it from thrombin unresponsive to responsive. Collectively, our finding demonstrate that the 8-bp Sp1 (5'-CCCCGCCC-3') can function as an essential part of a thrombin response element in the cox-1 promoter.

Effects of cellular expression of Sp1 on Cox-1 promoter activity

To determine ifelevated Sp1 expression is sufficient to increase Cox-1 promoter activity, BAEC cellc were transiently transfected with Cox-1 promoter vector and expression plasmids for Sp1 (Figure 5). Overexpression of Sp1 increased Cox-1 promoter activity nearly 3-fold in BAECs.

Mechanism of Cox-1 induction by thrombin

Finally, to elucidate the mechanism of Cox-1 by thrombin, we measured the level of the major AA metabolite, PGI2, secreted by BAECs. We observed a dose-dependent induction of PGI2 synthesis by thrombin (fig 6).

Discussion

It has been realized that thrombin has many functions distinct from the activation of the coagulation cascade. It is capable of transmitting intracellular signals and appears to participate in metastasis and the tumor-associated angiogenesis (24). Involvement of thrombin in angiogenesis has been reported to be associated with Cox-1 in the endothelial cells. For this reason, we were attempting to investigate the molecular mechanisms by which thrombin led to angiogenesis in endothelial cells. In the present study, we investigated the effects of thrombin on the Cox-1 expression, which is thought to be a major pathological change in tumor cells. The results demonstrated that thrombin potentiated cell growth and increased expression of the Cox-1 in cultured BAE cells.

Cyclooxygenase (Cox-1), also known as prostangandin (PG) H synthase, catalyzes the rate-limiting steps in the formation of prostangandin endoperoxide (25). The Cox enzyme possesses oxygenase and peroxidase activities and thus catalyzes the formation of PGH2 from arachidonic acid (9). Two Cox isoenzymes, designated as Cox-1 and -2 (26), exhibit similar enzymatic properties; however, the Cox-1 enzyme is ubiquitously expressed, whereas the Cox-2 isoenzyme is expresse as an immediate-early gene after stimulation by a wide variety of extracellular stimuli (27). Gene knock-out studies have suggested that Cox-1 and –2 isoenzymes possess distinct function (28-30). In addition, the Cox-2 enzyme is localized in the endoplasmic reticulum (ER) as well as the nuclear envelope, whereas the Cox-1 enzyme is localized primarily in the ER (31). It had been demonstrated that the rate- limiting enzyme in vascular PGI2 synthesis in newborn lung is cyclooxygenase (Cox), and that the capacity for vascular PGI2 production increase markedly during the early postnatal period. This is due to an upregulation in the expression of the

type 1 isoform of Cox (Cox-1) (32).Cox-1 plays an important role in angiogenesis and endothelial growth. But the mechanisms underlying its expression are not well understood. As the best we know, this is the first report to characterize the Cox-1promoter activity induced by thrombin. We identified the regions containing a positive regulatory element within the 5'-flanking region of the human Cox-1 gene. By deletion analysis, a 18-bp fragment within the 5'-flanking region of the Cox-1 promoter was identified, which appears to be essential for the cox-1 expression induced by thrombin. No significant changes were noted after deletion of the element from -1261 to +28, whereas deletion between –1261 ~ -565 reduced the promoter activities to 77% that of the whole promoter fragment. However, the promoter activity was reduced significantly when elements from –565 to –255 was removed. This region of the Cox-1 promoter only contains a putative Sp-1 binding site. Sp1 belongs to a zinc finger family of transcription factors that can activate transcription of a subset of genes containing Sp1 sites, including human tissue factor (33), human VEGF gene (34), and human 12 (s) lipoxygenase (35). Analysis by band shift assays of Sp1 binding to the Cox-1 gene had shown two bands were specifically competed by Sp1 or unlabeled probes but not by AP2 oligonucleotide. Furthermore, a 50-fold molar excess of –565/-255 in which the canonical Sp1 site was mutated failed to inhibit the formation of the two DNA-protein complexes. The band was supershifted with antibody directed against Sp1 but not with unrelated antibody such as anti-Cox-1 antibody. The present data suggest that Sp-1 may be necessary for up-regulation of the cox-1 promoter activity induced by thrombin.

Thrombin is an important growth factor and immuno-regulator for the tissue injury. Highly purified thrombin stimulates proliferation of chick embryo and mammalian fibroblasts under serum-free culture condition, and it activates monocytes, NK cells, T cells, and endothelial cells. Early studies showed that action at cell surface is sufficient for thrombin to stimulate cell proliferation by initiating transmembrane signals. Several laboratories have cloned members of a proteolytically active seven-transmembrane G-protein-linked receptor family that include PAR1, PAR2, PAR3, and PAR4 (36). These receptors are proteolytically activated by thrombin to generate a new NH2 terminus, which acts as a tethered ligand and promotes the interaction between the receptor and the G-proteins at the intracellular side of the membrane. To understand whether the thrombin-induced Cox-1 up-regulation was also through these pathways, we investigated effects of various inhibitors of the thrombin receptor. Here, we show that induction of Cox-1 by thrombin was inhibited by pretreatment with Hirudin, a thrombin receptor antagonist. This suggests that thrombin acts via activation of the receptor.

In summary, our studies firstly demonstrate that thrombin up-regulated of Cox-1 via a transcriptional level mechanism. We further demonstrated the critical role of the region from -115 to –97 of the Cox-1 promoter in mediating up-regulation of Cox-1 induced by thrombin, and in the basic Cox-1 promoter activity.. The exact mechanisms by which the region from -115 to -97 exerts its activity on the expression of Cox-1 remains unclear and is therefore the focus of our future study.

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