Induction of prostaglandin endoperoxide synthase 2 by mitogen-activated protein kinase cascades

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Prostaglandin endoperoxide synthase (PGHS) catalyses the rate-limiting step in the formation of prostaglandin and thromboxane eicosanoids from arachidonic acid released by phospholipase A_2. Two forms of PGHS exist, PGHS-1 and PGHS-2. PGHS-2, normally absent from cells, is rapidly expressed in response to a wide variety of stimuli and has been implicated in the pathogenesis of colon cancer and several inflammatory diseases. The three principal mitogen-activated protein kinase (MAPK) pathways are the extracellular signal-regulated protein kinase (ERK), the c-Jun N-terminal kinase (JNK) cascade and the p38-MAPK cascade. The present study was undertaken to investigate the putative involvement of the MAPK cascades in PGHS-2 induction. The potential role of ERK in PGHS-2 up-regulation was assessed by using cell lines expressing, both stably and after adenoviral infection, constitutively active forms of its upstream activator MAPK/ERK kinase (MEK1). The possible involvement of JNK and p38-MAPK in positively modulating PGHS-2 transcription was investigated by using adenovirus-mediated transfer of active forms of their respective specific upstream kinases, mitogen-activated protein kinase kinase (MKK) 7 and MKK3/MKK6. ERK activation promoted the induction of PGHS-2 mRNA and protein. Similarly, activation of JNK by Ad-MKK7D and p38-MAPK by Ad-MKK3bE/Ad-MKK6bE resulted in the increased expression of PGHS-2. These results provide evidence that activation of all three of the major mammalian MAPK leads to the induction of PGHS-2 mRNA and protein. Because PGHS-2 is up-regulated by a diverse range of stimuli, both mitogenic and stress-evoking, these results provide evidence that the convergence point of these stimuli could be the activation of one or more MAPK cascade(s).

Key words: colon cancer, inflammation, signal transduction, transcriptional up-regulation.

INTRODUCTION

Prostaglandin endoperoxide synthase (PGHS) is the enzyme that catalyses the first, rate-limiting step in the formation of prostaglandins from arachidonic acid released by phospholipase A_2 [1]. Two forms of PGHS have been characterized, PGHS-1 and PGHS-2. PGHS-2, normally absent from cells, is rapidly expressed in response to a wide variety of stimuli [1]. Expression of PGHS-2 is elevated in colonic tumours [2–4]; genetic evidence has been provided that PGHS-2 expression is causal in the development of colon polyps and carcinoma [5]. In addition, PGHS-2 has been implicated in both the promotion [6] and resolution [7] of inflammation.

It has been reported that the HCT-116 cancer cell line both expresses elevated levels of PGHS-2 in the basal condition and also has the capacity to drive the expression of a PGHS-2-promoter–luciferase construct [8]. This result lends support to the hypothesis that elevated PGHS-2 expression in colonic cancer might be due to dysregulated transcription and would suggest that a transcription factor aberrantly over-activated (or over-expressed) in carcinoma cells facilitates the abnormal expression of PGHS-2. Alternatively, a negative regulator of PGHS-2 transcription might be lost or non-functional in these cells. The promoter/enhancer region of the various mammalian PGHS-2 genes share a number of modulatory elements, including cAMP-response element (CRE), nuclear factor (NF)-IL6, NF-κB and activator protein 2 [9]. The first three of these consensus sequences have been implicated in ligand-dependent up-regulation of the human enzyme [9–12]; in addition it has been proposed that p53 might negatively regulate PGHS-2 expression by binding to the TATA sequence (located between -13 and -26 bp in the human gene) [13].

The three principal mitogen-activated protein kinase (MAPK) pathways are the extracellular signal-regulated protein kinase (ERK), the c-Jun N-terminal kinase (JNK) and the p38-MAPK cascades [14]. These cascades consist of a three-kinase module composed of the tertiary kinase or MAPK (ERK, JNK and p38-MAPK), activated by a MAPK/ERK kinase (MEK, also referred to as MAPK kinase or MKK), which in turn is activated by a MEK kinase (MEKK). ERK is activated by MEK, which is activated by Raf, the prototypic mammalian MEKK. JNK is activated by MKK4 and MKK7, and p38-MAPK by MKK4,

Abbreviations used: C/EBP, CCAAT-enhancer-binding protein; CRE, cAMP-response element; ERK, extracellular signal-regulated protein kinase; FBS, fetal bovine serum; HUVEC, human umbilical-vein endothelial cells; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; MOI, multiplicity of infection; NF, nuclear factor; PGHS, prostaglandin endoperoxide synthase; SV40, simian virus 40; tHMC, SV40-transformed human mesangial cells.

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MKK3 and MKK6 [14–20]. In addition, evidence exists that MKK3, MKK4, MKK6 and MKK7 can be activated by MEKK1–3 [14,16]. The ERK pathway is mobilized by a wide range of extracellular stimuli, including G-protein-coupled ligands, growth factors and cytokines; activation of this pathway is integral to the signalling of agents evoking cellular proliferation and differentiation. The stress-activated MAPK, JNK and p38-MAPK have been shown to have pivotal roles in cellular response to both extracellular and intracellular stress; in contrast with ERK, their activation generally promotes growth inhibition and induces apoptosis [14].

ERK, JNK and p38-MAPK are activated by many of the diverse stimuli capable of inducing PGHS-2 expression [14]. Moreover, the regions of the human PGHS-2 proximal promoter that have been shown to be involved in mediating the up-regulation of this enzyme bind a number of MAPK-activated transcription factors [9,14]. In the current study we therefore investigated the ability of the three principal MAPKs to drive PGHS-2 up-regulation. The putative role of ERK in mediating PGHS-2 transcription was studied by using cell lines expressing constitutively active forms of its upstream activator MEK1 (also known as MKK1) [14]. The possible modulation of PGHS-2 expression by JNK and p38-MAPK was investigated by means of adenovirus-mediated transfer of constitutively active MKK7 [15–18] and MKK3/MKK6 [19,20] constructs.

EXPERIMENTAL

Materials

All cell culture reagents and routine biochemicals were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Anti-MEK and anti-(phospho-MAPK) [anti-(phospho-JNK) and anti-(phospho-p38-MAPK)] antibodies were from New England Biolabs (Beverly, MA, U.S.A.). Anti-(PGHS-2) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Enhanced chemiluminescence (ECL®) kits were obtained from Amersham (Piscataway, NJ, U.S.A.). Protein determinations were performed by means of a bicinchoninic acid assay obtained from Pierce (Rockford, IL, U.S.A.). A plasmid containing a full-length 4.2 kb PGHS-2 insert was kindly provided by Dr Harvey Herschman (Los Angeles, CA, U.S.A.). The *MEK1* constructs used in adenoviral generation and NIH 3T3 cells expressing *MEK1* constructs were kindly provided by Dr Nathalie Ahn (Boulder, CO, U.S.A.).

Stable cell lines

Parental NIH 3T3 fibroblasts and stably transfected cell lines derived from these were propagated in Dulbecco's modified Eagle's essential high-glucose medium, supplemented with 10 % (v/v) heat-inactivated calf serum, 100 i.u./ml penicillin and 100 μg/ml streptomycin. Cell lines expressing the pSV2*neo* vector or pMM9 vector constructs encoding constitutively active MEK1 (ΔN3/S222D; deletion of a predicted α-helix encompassing residues 32–51 and replacement of the regulatory Ser-222 residue with Asp), wild-type MEK1 and catalytically inactive MEK1 (K97M) under the control of the Maloney sarcoma virus long terminal repeat are as described previously [21]. Cells were made quiescent for 24 h before experiments.

Recombinant adenoviral vectors

A second catalytically active MEK1 construct (ΔN3/S218E-S222D, achieved by the replacement of both regulatory residues, Ser-218 and Ser-222, with glutamic and aspartic residues respectively and the deletion of a predicted α-helix encompassing residues 32–51 [21]) was incorporated into the recombinant adenoviral vector AdMEK_{CA}. AdMEK_{CA} was constructed from the replication-deficient adenovirus type 5 (Ad5) with deletions in the E1 and E3 genes (Ad-d1327), and a plasmid containing Ad5 sequences from bp 22 to bp 5790 with a deletion of the E1 region from bp 342 to bp 3523, a polycloning site under the control of the CMV promoter, the mutated human MEK1 gene and the simian virus 40 (SV40) polyadenylation signal [22]. The recombinant adenoviral constructs encoding activated MKK6 (Ad-MKK6bE), MKK3 (Ad-MKK3bE) and MKK7 (Ad-MKK7D) have been described previously [23–25].

Adenoviral infection

Human umbilical-vein endothelial cells (HUVEC) were cultured in MCDB 105 medium containing 20 % (v/v) fetal bovine serum (FBS) and endothelial cell growth supplement (80 μg/ml). HUVEC were rendered quiescent overnight in MCDB medium containing 1 % (v/v) FBS. Cells were infected with Ad-MEK_{CA} and the non-transgenic control virus Ad-d1327 at a multiplicity of infection (MOI) of 60, essentially as described previously [22]. The adenovirus-mediated gene transfer of MEK1 was verified by Western blot analysis of cell lysates with anti-MEK antibodies and the concomitant activation of ERK by means of an immunocomplex assay. At various time points after infection, total RNA was isolated and Northern analysis for PGHS-2 mRNA was performed.

For experiments with Ad-MKK7D and Ad-MKK3bE/MKK6bE, SV40-transformed human mesangial cells (tHMC) [22,26] were rendered quiescent for 24 h before being infected with Ad-MKK7D at a MOI of 100, with Ad-MKK3bE/MKK6bE at a MOI of 50/50 or with a control adenovirus encoding β-galactosidase (Ad-LacZ) at a MOI of 100, as described previously [22]. After 36 h the cells were lysed and the resultant cell lysates were subjected to a Western blot analysis with phospho-specific anti-JNK and anti-(p38-MAPK) antibodies. Having thus confirmed adenoviral transfer and the consequent activation of JNK and p38-MAPK, Western blot analysis of PGHS-2 expression was performed.

RNA extraction and Northern blot analysis

Total cellular RNA was isolated by the acid guanidinium thiocyanate method [27] and analysed exactly as described elsewhere [28].

Western analysis and immunocomplex assays

Western blot analysis and MAPK immunocomplex assays were performed exactly as described previously [29]. For ERK assays, samples standardized for protein (400 μl, prepared as described above) were immunoprecipitated with a polyclonal antibody against p42 ERK [29]. After incubation with Protein A–Sepharose, samples were washed twice with lysis buffer and twice with kinase assay buffer (20 mM Hepes, pH 7.4, containing 10 mM $MgCl_2$ and 200 μM orthovanadate) before being resuspended in 60 μl of kinase buffer containing 0.25 mg/ml myelin basic protein, 50 μM ATP and 5 μCi of [γ-^{32}P]ATP. The reaction was initiated by incubation at 30 °C for 15 min and terminated by the addition of 60 μl of Laemmli buffer. The samples were then subjected to SDS/PAGE [12 % (w/v) gel], followed by autoradiographic development.

RESULTS AND DISCUSSION

Activation of each of the three major MAPK cascades (ERK, JNK and p38-MAPK) results in the mobilization of numerous transcription factors and the up-regulation of target genes [14]. Here we present results that support the hypothesis that the induction of PGHS-2 expression in response to a variety of stimuli might be secondary to the activation of one or more of the three major MAPK cascades.

Investigations employing pharmacological MEK inhibitors have provided evidence that the ERK pathway is involved in PGHS-2 induction [30,31]. In addition, it has been reported that the overexpression of both wild-type ERK-1 and ERK-2 potentiates v-Src-mediated activation of a murine PGHS-2 promoter construct and have speculated that that this might occur in an Elk-1-dependent manner [32]. The putative role of ERK in PGHS-2 induction was assessed in the current study by using both cell lines stably transfected, and primary cells adenovirally infected, with constitutively active *MEK1* constructs. The NIH 3T3 ΔN3/S222D cell line is invaluable for studying the downstream targets of ERK activation because this MAPK pathway is continuously activated in these cells, independently of extracellular stimuli. Cells stably expressing MEK ΔN3/S222D exhibit greatly enhanced basal ERK activity when compared with cells transfected with vector only (pSV2*neo*) or cells expressing wild-type or catalytically inactive (K97M) MEK1 ([21], and results not shown). Correspondingly, levels of PGHS-2 protein [as assessed by probing cell lysates normalized with regard to protein with anti-(PGHS-2) antiserum] in quiescent NIH 3T3 ΔN3/S222D cells were found to be elevated in comparison with cells transfected with vector only (pSV2*neo*) or cells expressing wild-type or catalytically inactive MEK1 (Figure 1). ΔN3/S222D 3T3 cells also demonstrated constitutive up-regulation of PGHS-2 mRNA (results not shown); however, to determine the time course of PGHS-2 mRNA induction in response to ERK activation, an adenoviral construct encoding a second catalytically active MEK1 mutant, ΔN3/S218E-S222D, was employed and this, together with a control adenovirus construct, was used to infect HUVEC. Adenovirus-mediated MEK1 expression was confirmed by Western analysis (Figure 2, left-hand top panel) and the activation of ERK in cells infected with Ad-MEK_{CA} was verified by immunocomplex assay (Figure 2, left-hand middle panel). In agreement with the results obtained from the ΔN3/S222D fibroblast cell line, adenovirus-mediated constitutively active MEK1 expression also led to increased PGHS-2 protein expression (Figure 2, left-hand bottom panel). Northern analysis with a full-length PGHS-2 cDNA probe revealed that, in comparison with control virus-infected HUVEC, cells transfected with Ad-MEK_{CA} exhibited an induction of PGHS-2 mRNA within 3 h of infection; this was increased at 6 and 9 h (Figure 2, right-hand panel).

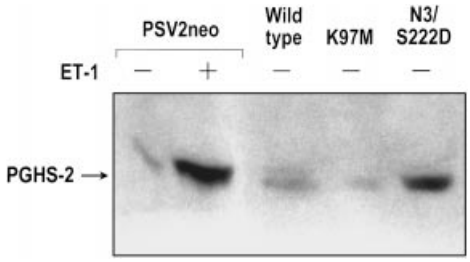

Figure 1 Fibroblasts transfected with the constitutively active MEK1 mutant demonstrate an increased level of PGHS-2 protein

pSVneo-transfected cells are shown unstimulated and after stimulation with endothelin-1 (ET-1; 100 nM, for 22 h). Quiescent cells stably expressing constitutively active MEK1 (ΔN3/S222D) demonstrated PGHS-2 expression at a level comparable to that induced in mock-transfected cells by ET-1. Shown is a representative experiment; the experiment was performed at least three times.

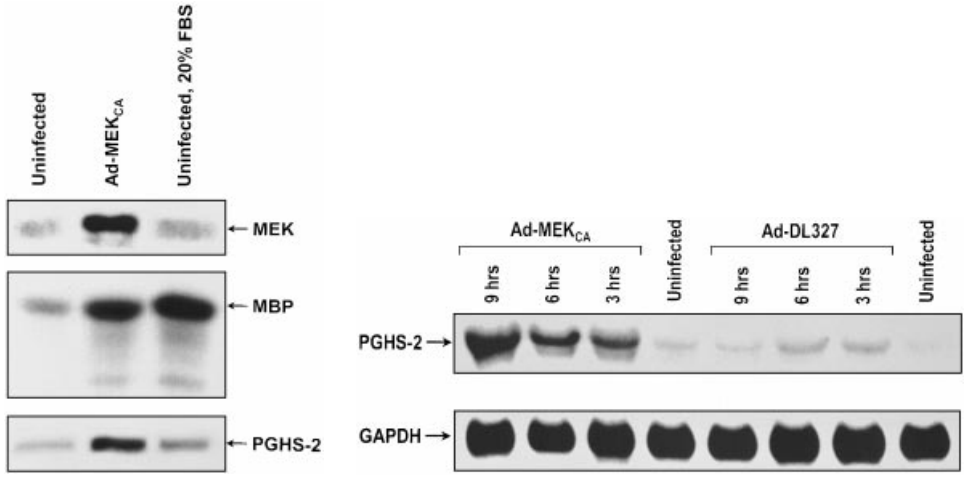

Figure 2 HUVEC infected with Ad-MEK_{CA} exhibit increased MEK protein expression, ERK activation, induction of PGHS-2 mRNA and PGHS-2 protein expression

Cell lysates of HUVEC infected with adenovirus encoding constitutively active MEK (N3/S218E-S222D) for 6 h (Ad-MEK_{CA}) or control cells (uninfected) were subjected to SDS/PAGE and Western blot analysis with anti-MEK antibodies (left-hand top panel). Lysates were subjected to a confirmatory immunocomplex assay for ERK activity (left-hand middle panel). As a positive control, uninfected cells were stimulated with 20% (v/v) FBS for 5 min (uninfected, 20% FBS). The positions of MEK1 and MBP (myelin basic protein) are indicated. Expression of PGHS-2 in these cell lysates was determined by Western analysis with anti-(PGHS-2) antiserum (left-hand bottom panel). At the various time points shown after the infection of HUVEC with AdMEK_{CA} or control Ad-DL327 (lanes corresponding to cells that received no adenovirus are indicated as uninfected), total cellular RNA was isolated and Northern blot analysis was performed with PGHS-2 cDNA (right-hand upper panel). After stripping of the membrane, it was rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to confirm even loading of RNA (right-hand lower panel).

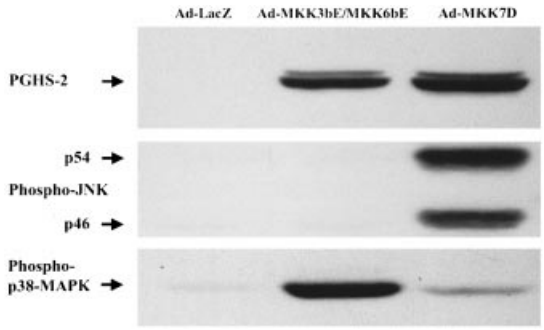

Figure 3 Stress-activated MAPK activation and PGHS-2 expression are increased after infection of tHMC with Ad-MKK3bE/Ad-MKK6bE and Ad-MKK7D

Cell lysates of tHMC infected with Ad-LacZ, Ad-MKK3bE/Ad-MKK6bE or Ad-MKK7D for 36 h were subjected to SDS/PAGE and Western blot analysis with anti-(PGHS-2) antibodies (top panel), anti-(phospho-JNK) antibodies (middle panel) or anti-(phospho-p38-MAPK) antibodies (lower panel). The positions of PGHS-2, phospho-JNK and phospho-p38-MAPK are indicated. Shown is a representative experiment; the experiment was repeated twice with similar results.

These results indicate that the sustained activation of ERK by two distinct catalytically active MEK1 mutants results in the up-regulation of PGHS-2 in both fibroblasts and HUVEC; taken together, the results support a role for activated ERK in the transcriptional up-regulation of PGHS-2. In addition to Elk-1, ERK has also been reported to phosphorylate and activate NF-IL6 [also known as CCAAT-enhancer-binding protein (C/EBP) β] [33]. Although the consensus motif for this transcription factor has been shown to be involved in the induction of human PGHS-2, this seems to be mediated via the binding of C/EBPδ [10]. NF-IL6 has been shown to regulate c-Fos expression [34] and thus, like Elk-1, might well have a role in the potentiation of PGHS-2 expression. The present results provide novel evidence *in vitro* that activated ERK is capable of not only potentiating [32] but actually effecting PGHS-2 transcription.

To assess the putative involvement of the stress-activated MAPK in the modulation of PGHS-2 expression, adenoviral constructs encoding constitutively active variants of their respective specific activating kinases were employed. MKK7 has been demonstrated to be a specific activator of the JNK family kinases [15–18], whereas p38-MAPK is the principal target of MKK3 and MKK6 [19,20]. tHMC were infected with equal adenoviral titres of Ad-MKK7D or a combination of Ad-MKK3bE and Ad-MKK6bE. Although Ad-MKK3bE and Ad-MKK6bE can each activate p38-MAPK independently, by using a combination of the two adenoviral constructs the possibility of isoform-specific activation of p38-MAPK by one or other of these MKKs is overcome [24]. Successful adenoviral transfer and activity were confirmed by means of Western blot analysis of phosphorylated, and hence activated, JNK and p38-MAPK by using phospho-specific antibodies (Figure 3). As can be seen, infection with Ad-MKK7D led to a marked activation of both the p46 and p54 JNK isoforms but had virtually no effect on the activation status of p38-MAPK (Figure 3, middle and bottom panels). Similarly, infection with Ad-MKK3bE/Ad-MKK6bE resulted in a significant activation of p38-MAPK without having any effect on either JNK isoform (Figure 3, middle and bottom panels). Cells transduced with Ad-LacZ exhibited no activation of either JNK or p38-MAPK (Figure 3, middle and bottom panels) and none of the three adenoviral constructs was found to activate ERK (results not shown).

Having thus confirmed the substrate specificity of the adenoviral constructs, the expression of PGHS-2 in tHMC cell lysates was also assessed by Western blotting. Infection of tHMC with either Ad-MKK7D or an Ad-MKK3bE/Ad-MKK6bE combination (with the consequent constitutive activation of JNK and p38-MAPK respectively) led to a marked increase in the expression of PGHS-2, whereas infection with Ad-LacZ had no effect on PGHS-2 protein levels (Figure 3, top panel). These results confirm that both stress-activated MAPKs are capable of driving PGHS-2 transcription. It has been reported that MEKK-1-dependent JNK activation results in the activation of a murine PGHS-2 promoter construct [32,35]. Guan et al. [36] reported that activation of the MEKK-1/MKK4 module up-regulates PGHS-2 expression via the concomitant activation of JNK and p38-MAPK; interestingly, they also found that the over-expression of a constitutively active MEKK-1 mutant resulted in the activation of MKK4 but not MKK3 or MKK6. Significantly, in the present study, novel evidence is given that the stress-activated MAPKs can each independently drive PGHS-2 expression and that this occurs in a MKK7-dependent and MKK3/6-dependent manner.

JNK phosphorylates c-Jun on its N-terminal activating domain [37], thereby inducing the formation of c-Jun homodimers and c-Jun–c-Fos heterodimers and leading to up-regulated transcription activity of numerous genes via activator protein 1 and CRE sites [38,39]. Studies have indicated that the CRE site of human PGHS-2 is involved in C/EBP-mediated up-regulation [10] and it has been reported that this site also facilitates the c-Jun-driven transcription of human and murine PGHS-2 [32,35,40]. Similarly, although p38-MAPK has been reported to activate a number of transcription factors [14] and to be involved in PGHS-2 up-regulation [36], the precise mechanism by which this MAPK facilitates PGHS-2 expression is as yet undetermined.

With regard to the potential role of MAPK cascades in effecting constitutive PGHS-2 expression in colonic cancer, ERK activation has been paralleled with tumorigenic progression in rat intestinal neoplasia [41] and it has been reported that pharmacological MEK inhibition significantly suppresses colonic tumour growth *in vitro* and *in vivo* [42]. In contrast, Wang and co-workers concluded that the ERK1/2 activity was down-regulated in colorectal tumour in comparison with adjacent normal tissue [43], and other investigators have correlated decreased ERK expression and activity with the progression of normal gastric tissue to adenoma [44,45]. Although our own investigations have confirmed tumour-associated PGHS-2 expression, we did not detect enhanced ERK activation in human colon cancer samples in comparison with control tissue (results not shown). In addition, although tumour tissue from five of eight patients exhibiting neoplasia-associated PGHS-2 had increased levels of JNK activity, and p38-MAPK displayed a tentative trend towards tumour-associated phosphorylation (results not shown), these results were far from unequivocal. Although JNK activation has been paralleled with tumorigenic progression in rat intestinal neoplasia [41], Wang and co-workers have reported that the activities of both JNK1 and p38-MAPK were in fact down-regulated in colorectal tumours in comparison with normal tissue [43]. It therefore seems that the role of ERK, JNK and p38-MAPK in the pathogenesis of colon cancer is still unclear and that, by extrapolation, so too does the involvement of these MAPKs in driving tumour-associated PGHS-2 expression.

In conclusion, the findings of the present study provide evidence that MAPK cascades are the convergence point of the numerous diverse stimuli that have been reported to up-regulate PGHS-2. However, although the ligand-dependent activation of

one or more of these cascades might drive pathogenic expression of PGHS-2 in, for example, inflammatory models [46], the role of MAPK in mediating colon-cancer-associated expression of this enzyme remains to be established.

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