Transcription-based COX-2 inhibition: A therapeutic strategy

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Summary
Potent selective cyclooxygenase-2 (COX-2) inhibitors are effective in controlling inflammatory disorders but are associated with cardiovascular complications. Their clinical use has been severely limited. We propose that transcription-based inhibition of COX-2 expression represents a therapeutic strategy that may circumvent the undesired complications of COX-2 inhibitors. Reported data from several laboratories including ours have identified C/EBPβ as a key transactivator mediating COX-2 transcriptional activation induced by diverse pro-inflammatory mediators. Results from our recent work show that sodium salicylate at pharmacological concentrations inhibits C/EBPβ binding to COX-2 promoter by direct inhibition of p90 ribosomal S6 kinase (RSK). RSK phosphorylates C/EBPβ and stimulates its binding to enhancer elements. We propose that RSK1/2 is a potential target for screening drugs with novel anti-inflammatory and anti-neoplastic therapeutic potentials.

Keywords
Cyclooxygenase-2, inflammation, C/EBPβ, aspirin, P90 ribosomal S6 kinase

Introduction
Cyclooxygenase-2 (COX-2) is expressed at a very low level in most human cells at basal state and is highly inducible by diverse pro-inflammatory mediators including cytokines, lipopolysaccharide (LPS) and mitogenic factors (1). These factors induce robust COX-2 expression at the transcriptional level involving several key transactivators that bind to the promoter-enhancer region situated within 1 kb upstream of the transcription start site (2, 3). The induced COX-2 is primarily localized to the lumen of endoplasmic reticulum where it is coupled to phospholipase A2 (PLA2) and a downstream enzyme such as prostacyclin synthase (PGIS) (4). PLA2 catalyzes the release of free arachidonic acid (AA) from membrane phospholipids. AA enters COX-2 where it is converted to PGG2 by cyclooxygenase activity and PGG2 is reduced to PGH2 by peroxidase activity (5). PGH2 is a common substrate for prostanooid production. For example, it is converted to PGE2 by prostaglandin E synthase (PGES) and PGI2 by PGIS. It has been suggested that COX-2 is co-induced with the microsomal (membrane-bound) PGES isoform in inflammatory cells which results in robust synthesis of PGE2, an important pro-inflammatory mediator (6). In vascular endothelial cells, COX-2 is co-localized with PGIS which results in abundant PGI2 production (4). At physiological concentrations, PGI2 plays an important role in maintaining vascular tone, vascular interaction with blood cells and protecting vascular cells from apoptosis (7, 8). There is strong evidence that COX-2/mPGES plays a major role in inflammatory joint disease and vascular inflammation (6). Selective COX-2 inhibitors such as rofecoxib and celecoxib prove to be effective in controlling joint inflammation and were widely used by a large population. Unfortunately, the coxibs inhibit not only the pro-inflammatory COX-2/PGES pathway in inflammatory cells but also the protective COX-2/PGIS pathway in vascular cells. It has been proposed that the cardiovascular complications now well documented among coxib users are due to inhibition of COX-2 derived PGI2 by vascular endothelial cells (9–14). It should be noted that COX-2 derived PGI2 plays important physiological roles in cells other than the vascular cells. It has been shown that COX-2 derived PGI2 is crucial for in-vitro embryo development and uterine implantation (15, 16). COX-2 derived PGI2 protects renal interstitial cells from apoptosis by hypertonicity and myocardial cells from damage by doxorubicin (17, 18). Long-term use of potent selective COX-2 inhibitors may therefore be associated with serious adverse effects besides the well recognized cardiovascular complications.
Since transcriptional induction of COX-2 expression by pro-inflammatory mediators appears to depend on transactivators known to be involved in inflammation and tissue injury and the mechanisms by which the pro-inflammatory mediators induce binding of these transactivators to their respective binding sites are extensively elucidated, it may be feasible to develop therapeutic strategies centering on the inhibition of the DNA binding activity of the pro-inflammatory transactivators. An important aspect of this therapeutic approach is that COX-2 transcriptional activation by various pro-inflammatory mediators requires binding of multiple transactivators to the promoter and inhibition of the binding of a transactivator results in partial suppression of COX-2 expression. Importantly, it may be possible to design transcription-based control of COX-2 by targeting a biochemical process selectively used by pro-inflammatory mediators. Here, we will review the role of CCAAT/enhancer binding protein (C/EBP) in COX-2 transcription induced by common pro-inflammatory mediators and present evidence for the involvement of p90 ribosomal S6 kinase (RSK) in inducing C/EBPβ binding to COX-2 promoter and activating COX-2 promoter function. We will also review the involvement of C/EBP in constitutive COX-2 overexpression in certain types of cancer cells.

**Transcriptional activation of COX-2 by pro-inflammatory mediators**

COX-2 promoters have been extensively characterized in murine and human cells (19–22). The promoter and enhancer regions of COX-2 from murine, bovine and human sources are similar with only minor differences. COX-2 promoters harbor a canonical TATA motif and several functionally important enhancer elements within ~500 bp upstream of the transcription start site (2, 3). A cyclic AMP response element (CRE) located at −53/−59 is absolutely essential for basal and induced COX-2 transcription as mutation of this site renders COX-2 promoter completely silent and unable to respond to exogenous stimuli (21, 24). Reported data have shown that IL-1β, LPS, TNFα, or PMA stimulate COX-2 promoter activity by inducing binding of multiple transactivators to their respective binding sites, notably AP-1 site located close to CRE, C/EBP site (NF-IL6) located at −132/−124 and two κB sites located at −213/−222 and −447/−438. A number of transactivators have been shown to be involved in COX-2 transcriptional activation by cytokines, LPS and growth factors. They include C-Jun/C-Fos (AP-1), p65/p50 NF-κB, C/EBPβ and C/EBPβ, NF-AT, SP-1, CREB-2/ATF2 (25–32). A pro-inflammatory mediator generally elicits the binding of a specific set of transactivators to the enhancer elements which in turn recruit 300/CBP co-activator (23). Among the transactivators, C/EBPβ plays a crucial role in COX-2 expression in human and murine cells induced by cytokines, PMA, abnormal shear stress and LPS (26, 28, 33–36). C/EBPβ belongs to the basic leucine zipper C/EBP family that comprises six members. C/EBPβ shares sequence homology and functional properties with C/EBPδ and C/EBPα. C/EBPβ has several truncated forms due to use of alternate translation codon ATG as a result of ribosomal translation leakage (33, 38). A near full-length truncated form called LAP is transcriptionally active and is the main transactivator for COX-2, and a short form, LIP, is expressed and serves as a dominant negative form (39). PMA induces binding of full-length as well as LAP and LIP to the C/EBP site suggesting a dynamic regulation of C/EBPβ transactivator activity by LIP (39). C/EBPδ constitutively binds C/EBP enhancer element. PMA treatment reduced C/EBPδ binding and replaced it with C/EBPβ binding. Thus, C/EBPβ binding to the COX-2 promoter is dynamically regulated. As will be described in more detail in the next sections, we consider C/EBPβ as a potential target for transcription-based COX-2 suppression because of its common involvement in transactivating COX-2 promoter induced by pro-inflammatory mediators and its intrinsic dynamic regulation. Furthermore, its binding is activated by phosphorylation. By identifying the kinase(s) that phosphorylates and activates C/EBPβ binding, it will be possible to use high throughput techniques to screen compounds that block C/EBPβ-mediated COX-2 expression.

**Roles of C/EBPβ in constitutive COX-2 transcriptional activation in cancer cells**

COX-2 is constitutively overexpressed in cancer cells (40–42). Its overexpression plays an important role in cancer growth and metastasis (43, 44). COX-2 promoter is constitutively activated in several types of cancer cells including colon and skin cancer cells. It has been shown that transfection of colon cancer cells (HCT-116) with a COX-2 promoter constructed into a luciferase expression vector results in constitutive expression of luciferase consistent with the presence in colon cancer cells of an active transactivation program (45). C/EBP and CRE enhancer elements in the COX-2 promoter/enhancer region were identified as being crucial for constitutive COX-2 transcriptional activation in colon cancer cells. The transactivators that bind these enhancer elements and activate COX-2 transcription have not been clearly defined. It has been reported that COX-2 transcription in colon cancer cells depends on ERK (46). As ERK and its downstream RSK are capable of phosphorylating and activating C/EBPβ binding to COX-2 promoter, it is likely that C/EBPβ plays a crucial role in COX-2 transcriptional activation in colon cancer cells.

COX-2 promoter has been shown to be constitutively activated in a skin cancer cell line (47). E-box and C/EBP enhancer elements have been shown to be essential for constitutive promoter activation. DNA-protein binding analysis by electrophoresis mobility shift assay shows binding of C/EBPβ and C/EBPδ to the C/EBP site and E-box of COX-2 promoter (47). Taken together, these data suggest that C/EBPβ binding to a specific C/EBPδ enhancer element located close to the TATA box region plays an essential role in constitutive COX-2 transcriptional activation in colon and skin cancers and induced COX-2 transcriptional activation by pro-inflammatory mediators in normal inflammatory cells.
Salicylate inhibits COX-2 expression by targeting RSK-activated C/EBPβ binding

The anti-inflammatory action of salicylate was recognized over a century ago, but the mechanism by which it controls inflammation remains to be elucidated. Besides being a natural product from diverse plants, salicylate is a key metabolite of aspirin in vivo. Salicylate is considered to contribute to the anti-inflammatory actions of aspirin. It is, therefore, of considerable importance to know how it works. Salicylate has very weak inhibitory action on COX-2 or COX-1 catalytic activity. Its anti-inflammatory action is unlikely due to blocking the COX-2 catalytic activity. It has been suggested that salicylate may exert its action by inhibiting DNA binding activity of transactivators that mediate the transcription of pro-inflammatory genes (48–50). Results from our laboratory indicate that salicylate at pharmacological concentrations (10 μM to 10 μM) inhibits COX-2 expression in human endothelial cells and fibroblasts induced by PMA, IL-1β and LPS (51, 52). Salicylate inhibits COX-2 transcriptional activation by targeting C/EBPβ transactivator. PMA, IL-1β and LPS induce C/EBPβ binding to a specific binding site (-132 to -124) on human COX-2 promoter. Inhibition of C/EBPβ binding results in reduction of COX-2 protein levels by about 50% (51, 52). C/EBPβ also plays a crucial role in transcriptional activation of inflammatory cytokines and other inflammatory genes (38). To test the hypothesis that salicylate is capable of inhibiting other C/EBPβ-dependent genes, we investigated the effect of salicylate on inducible nitric oxide synthase (iNOS) expression in RAW 264.7, a murine macrophage. As COX-2 is also transcriptionally activated by LPS in this cell line, we also evaluated the effect of salicylate on COX-2 as a reference. The results show that salicylate inhibited LPS-induced iNOS and COX-2 expression at the transcriptional level through inhibition of C/EBPβ binding to their binding sites on iNOS and COX-2 promoters, respectively. Surprisingly, salicylate inhibited iNOS transcription induced by LPS and interferon-γ (IFNγ) only at 4 hours (h) after stimulation but not at 8 h or 24 h (53). The reason for the time-dependent differential inhibition of iNOS transcription is unclear but might be due to complex LPS/IFNγ-induced signaling pathway changes at different time periods after stimulation.

C/EBPβ at the resting state has weak DNA binding activity due to an intramolecular inhibitory element. Upon cellular activity, C/EBPβ may be phosphorylated by a number of kinases (54–57). C/EBPβ phosphorylation probably releases the intramolecular inhibition and exposes the DNA binding site. Our results have shown that PMA-induced C/EBPβ binding is mediated by RSK. By using biochemical and molecular genetic approaches, we show that RSK-2 phosphorylates Thr-266 of C/EBPβ (58). A dominant negative RSK-2 abrogates PMA-induced C/EBPβ binding and PMA-induced COX-2 expression. To determine whether salicylate inhibits RSK activity, we transfected fibroblasts with a FLAG-tagged RSK. Following treatment with PMA for 4 h, cells were lysed and RSK activity was measured. Salicylate inhibited PMA-induced RSK activity. To determine whether salicylate directly inhibits RSK, cells were lysed and RSK was purified by FLAG-affinity column. The purified RSK was incubated with sodium salicylate at increasing concentrations. Salicylate inhibited RSK activity in a concentration-dependent manner (58). These results indicate that salicylate has a direct inhibitory action on RSK1/2. As RSK is phosphorylated by ERK, and ERK has been shown to be capable of direct phosphorylation of C/EBPβ, we determined whether salicylate inhibits ERK activity. The results show that salicylate had no effect on ERK. These results suggest that salicylate selectively inhibits RSK and thereby suppresses RSK-mediated C/EBPβ binding. As RSK is involved in diverse pathophysiological processes including inflammation and diabetes (59, 60), and C/EBPβ is a crucial transactivator for transcriptional activation of cytokines, iNOS and other pathophysiologically important genes (38), salicylate may have a broad effect on diverse inflammatory disorders through its inhibition of RSK activity and C/EBPβ binding.

An array of polyphenolic compounds purified from plants, fruits, tea and herbs has been shown to suppress COX-2 and iNOS transcriptional activation induced by pro-inflammatory mediators and mitogen factors (61, 62). These compounds are active antioxidants as well. It is unclear how they suppress COX-2 expression. It would be interesting to test whether these purified compounds have a similar effect as salicylate on RSK activity and the consequent suppression of C/EBPβ DNA binding. It has been suggested that some of the compounds may inhibit NF-κB binding. Further studies are needed to elucidate the mechanisms by which the natural products inhibit COX-2 and iNOS. These studies will identify common targets that are valuable for transcription-based drug discovery.

Transcription-based COX-2 inhibition as a potential therapeutic strategy

As inflammation-mediated COX-2 transcriptional activation requires distinct transactivators such as C/EBPβ, NF-κB and C-Fos/C-Jun (AP-1), it is possible to design inhibitors to block selectively inflammation-coupled COX-2 expression. As illustrated in Figure 1, there are several potential therapeutic approaches such as suppressing the expression of C/EBPβ by small interference RNA (siRNA) or blocking the activation of C/EBPβ by targeting kinases that are responsible for inducing C/EBPβ DNA binding. Our study suggests that RSK is a potential target for developing transcription-based therapy. RSK comprises a large family of proteins among which RSK-1 and RSK-2 (RSK1/2) share a high degree of sequence homology and overlapping biochemical and functional properties (63). Although RSK1/2 is a major downstream enzyme of p42/p44 mitogen-activated protein kinase (MAPK or ERK), it is activated by other upstream kinases such as protein kinase C (PKC) (64, 65). RSK1/2 phosphorylates serine or threonine residues of several transactivators including C-Fos (66, 67). RSK phosphorylates IκBα and stimulates its degradation, thereby activating NF-κB (68, 69). RSK1/2 has been implicated in diabetic myocardial ischemic injury (59) and prostate cancer cell proliferation (60). Our results have shown that RSK1/2 is pivotal in COX-2 transcription induced by pro-inflammatory mediators (58). Inhibition of RSK1/2 may not only block COX-2 transcriptional acti-
C/EBPβ is essential for COX-2 transcription induced by diverse pro-inflammatory mediators. Furthermore, it plays a key role in mediating constitutive overexpression in cancer cells. There are several C/EBPβ isoforms which are derived from alternate translation start site due to ribosomal leakage (70). Functionally, the LAP isoform mediates C/EBPβ transactivation activity while LIP, a short isoform, blocks the transactivation activity of LAP (39, 70). There may be several approaches to control C/EBPβ binding activity. As has been described above, one approach is to inhibit kinase that phosphorylates and induces C/EBPβ DNA binding. In addition to inhibiting RSK1/2 activity, targeting other kinases such as CaMK IV may have therapeutic value. Another approach is to develop agents that directly inhibit C/EBPβ binding. One candidate is the dominant-negative LIP. LIP is capable of binding C/EBPβ (LAP) and C/EBPδ and preventing DNA binding of these C/EBP isoforms.

NF-κB plays an essential role in COX-2 and iNOS transcriptional induction by diverse pro-inflammatory mediators, notably tumor necrosis factor-α (TNF-α) (22). NF-κB comprises five isoforms, two in the NF-κB family (NF-κB1 also known as p105/p50 and NF-κB, known as p100/p52) and three in the Rel family (C-Rel or p75, RelA or p65 and RelB or p68). These isoforms form homodimers or heterodimers (71). All isoforms are sequestered in cytosol by binding to an inhibitor known as IκB (72). Upon activation, IκB is phosphorylated by a number of kinases including IκB kinases and RSK1/2 which facilitates IκB ubiquitination and degradation (68, 69). Free NF-κB translocates to the nucleus and binds to specific promoter enhancer elements. To suppress NF-κB transactivation actions, one target is the kinase(s) that phosphorylates IκB. Compounds to inhibit NF-κB are being developed. Other important transcription targets include AP-1, which is a heterodimer of C-Fos and C-Jun family proteins. As the approaches are in principle similar to these for C/EBPβ and NF-κB, they will not be described in detail here.

One caveat of the transcription-based COX-2 inhibition is the lack of specificity. For example, RSK1/2 is involved in phosphorylating diverse proteins, some of which may exert beneficial physiological functions such as cell survival (67). Inhibition of RSK may therefore shift the balance between pro- and antiapoptotic factors in cells.

Conclusion and perspective

Transcription-based COX-2 inhibition represents a useful therapeutic strategy to treat COX-2-mediated inflammatory disorders and tissue injury. As COX-2 transcription is coupled to pro-inflammatory stimuli, it is possible to selectively block inflammation-stimulated COX-2 expression without suppressing the physiological function of COX-2. Our results have shown that RSK1/2 induces C/EBPβ binding, thereby activating COX-2 transcription, and salicylate suppresses C/EBPβ-mediated COX-2 expression by inhibiting RSK1/2. Based on these results, we propose that RSK1/2 is a target for drug screening and discovery. Future plans include developing high-throughput techniques for screening small-molecular-weight compounds and selecting suitable compounds for animal and human studies.
References


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