Proceedings of International Symposium on Recent Advances in Molecular Markers for Carcinogenesis and Chemoprevention (May 3, 2000, Seoul, Korea)

SUPPRESSION OF PHORBOL ESTER-INDUCED EXPRESSION OF CYCLOOXYGENASE-2 AND INDUCIBLE NITRIC OXIDE SYNTHASE BY SELCTED CHEMOPREVENTIVE PHYTOCHEMICALS VIA DOWN-REGULATION OF NF-κB

Young-Joon Surh

College of Pharmacy, Seoul National University, Seoul, Korea

SUPPRESSION OF PHORBOL ESTER-INDUCED EXPRESSION OF CYCLOOXYGENASE-2 AND INDUCIBLE NITRIC OXIDE SYNTHASE BY SELECTED CHEMOPREVENTIVE PHYTOCHEMICALS VIA DOWN-REGULATION OF NF-κB*

Young-Joon Surh

College of Pharmacy, Seoul National University, Seoul 151-742

SUMMARY A wide array of naturally occurring substances, particularly those present in dietary and medicinal plants, have been reported to possess substantial cancer chemopreventive properties. Certain phytochemicals retain strong antioxidative and anti-inflammatory properties which appear to contribute to their chemopreventive or chemoprotective activities. Inducible cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) are important enzymes that mediate inflammatory processes. There is some evidence that expression of both COX-2 and iNOS is co-regulated by the eukaryotic transcription factor NF-κB. Increased expression of COX-2 and/or iNOS has been associated with pathophysiology of certain types of human cancers as well as inflammatory diseases. Since inflammation is closely linked to tumor promotion, substances with potent anti-inflammatory activies are anticipated to exert chemopreventive effects on carcinogenesis, particularly in the promotion stage. An example is curcumin, a yellow pigment of turmeric (*Curcuma longa* L., Zingiberaceae), that strongly suppresses tumor promotion. In the present work, we have found that some naturally occurring diaryl heptanoids structurally related to curcumin have substantial anti-tumor promotional activities in two-stage mouse skin carcinogenesis. Thus, yakuchinone A [1-(4'-hydroxy-3'-methoxyphenyl)-7-phenyl-3-heptanone] and yakuchinone B [1-(4'-hydroxy-3'-methoxyphenyl)-7-phenylhept-1-en-3-one] present in *Alpinia oxyphylla* Miquel (Zingiberacease) attenuate phorbol ester-induced inflammation and papilloma formation in female ICR mice. These diarylheptanoids also suppressed phorbol ester-induced activation of epidermal ornithine decarboxylase and its mRNA expression when applied onto shaven backs of mice. Yakuchinone A and B as well as curcumin inhibited phorbol ester-induced expression of COX-2 and iNOS and their mRNA in mouse skin via inactivation of NF-κB. Capsaicin, a major pungent ingredient of red pepper also attenuated phorbol ester-induced NF-κB activation. Similar suppression of COX-2 and iNOS and down-regulation of NF-κB activation for its DNA binding were observed with the ginsenoside Rg_3 and the ethanol extract of *Artemisia asiatica*. We have also found that certain anti-inflammatory phytochemicals exert inhibitory effects on phorbol ester-induced COX-2 expression and NF-κB activation in immortalized human breast epithelial (MCF-10A) cells in culture. One of the plausible mechanisms underlying inhibition by aforementioned phytochemicals of phorbol ester-induced NF-κB activation involves interference with degradation of the inhibitory unit, IκBa, which blocks subsequent nuclear translocation of the functionally active p65 subunit of NF-κB. The activation of epidermal NF-κB by phorbol ester and subsequent induction of COX-2 hence appear to play an important role in intracellular signaling pathways leading to tumor promotion and targeted inhibition of NF-κB may provide a new promising cancer chemopreventive strategy.

*A preliminary account of part of this work has been presented at the 91st annual meeting of the American Association for Cancer Research held in San Francisco on April 1-5, 2000.

Elevated levels of prostaglandins and enhanced cyclooxygenase (COX) activities have been often observed in various cancers of epithelial origin (Dubois *et al.*, 1998; Rigas *et al.*, 1993). Prostaglandins, especially prostaglandin E_2, affect cell proliferation and tumor growth and also suppress the immune response to malignant cells (Earnest *et al.*, 1992; Marnett, 1992). Therefore, high levels of prostaglandins could favor the growth of malignant cells by increasing cell proliferation, promoting angiogenesis, and inhibiting immune surveillance. Two isozymes, designated COX-1 and COX-2, have been identified. COX-1 is a house-keeping enzyme that is expressed in most tissues and catalyzes the synthesis of prostaglandins for normal physiological functions. In contrast, COX-2 is rapidly induced by external stimuli, including tumor promoters, growth factors, and cytokines (Crofford *et al.*, 1994; Inoue *et al.*, 1995) (Figure 1). Cells which are actively involved in mediating inflammatory responses, such as macrophages, endothelial cells and fibroblasts, highly express the *cox*-2 gene (Hla *et al.*, 1993). Considerable evidence has accumulated to support that COX-2 is important for carcinogenesis. For example, COX-2 is up-regulated in transformed cells (Kutchera *et al.*, 1996; Sheng *et al.*, 1997; Subbaramaiah *et al.*, 1996) and various forms of cancer (Kargman *et al.*, 1995; Muller-Decker *et al.*, 1995; Ristimaki *et al.*, 1997), whereas levels of COX-1 remain essentially unchanged. A null mutation for *cox*-2 markedly reduced the number and the size of intestinal tumors in a murine model of familial adenomatous polyposis (Oshima *et al.*, 1996). In addition, a selective inhibitor of COX-2 caused nearly complete suppression of azoxymethane-induced colon cancer (Kawamori *et al.*, 1998). Overexpression of COX-2 blocked apoptosis and increased the invasiveness of malignant cells (Tsujii *et al.*, 1997; Tsujii and DuBois, 1995). These effects were reversed by sunlindac sulfide. Based on these findings, it is conceivable that targeted inhibition of COX-2 is a promising approach to prevent cancer (Earnest *et al.*, 1992; Kawamori *et al.*, 1998).

Nitric oxide synthase (NOS) is another important enzyme which is involved in inflammation and other human disorders. Nitric oxide (NO) is generated via oxidation of the terminal guanidino nitrogen atom of L-arginine by NOS. NO is released during a variety of pathophysiological responses including circulatory shock, inflammation (Ohshima *et al.*, 1994) and carcinogenesis (Yun *et al.*, 1996). Molecular cloning and sequencing analyses revealed the existence of at least three main types of NOS isozymes. Both neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, whereas synthesis of inducible isoform (iNOS) is transiently stimulated in response to bacterial lipopolysaccharide and pro-inflammatory cytokines (e.g., TNF-α) and hence considered to be a major isozyme responsible for the production of NO upon inflammatory stimuli (Szabo, 1995; Yun *et al.*, 1996). NO has been proposed to be an important mediator of

tumor growth. For instance, endogenously formed NO appeared to cause the neoplastic transformation of C3H10T1/2 mouse fibroblasts (Mordan *et al.*, 1993). Both constitutive and inducible forms of NOS have been detected in human breast tumors (Thomsen *et al.*, 1995), cervical tumors (Thomsen *et al.*, 1994), tumors associated with the central nervous system (Cobb *et al.*, 1995), colon cancer (Radomski *et al.*, 1991), and head and neck cancers (Gallo *et al.*, 1998; Rosbe *et al.*, 1995).

The NOS and COX pathways share a number of similarities. Under normal circumstances, the constitutive isoforms of COX and NOS are found in virtually all organs. They regulate several important physiological effects (e.g., antiplatelet activity, vasodilation, cytoprotection, etc.). On the other hand, in inflammatory setting, NOS and COX are induced in a variety of cells, resulting in the production of large amounts of proinflammatory and cytotoxic NO and prostaglandins respectively.

Nuclear factor-κB (NF-κB) is a ubiquitous, pleiotropic, multi-subunit eukaryotic transcription factor consisting of either homo- or heterodimers of various subunits of Rel family proteins referred to as p50, p52, p65 (RelA), c-Rel, and Rel-B (Blank *et al.*, 1992; Grilli *et al.*, 1993; Schmitz *et al.*, 1995). These complexes are activated by antigens, viruses, bacteria, and inflammatory cytokines, and result in transcriptional initiation of a diverse set of genes whose products are important in the immune and inflammatory responses (Kopp and Ghosh, 1995). The importance of NF-κB in mediating inflammatory events is also evident from experiments utilizing knock-out animals, suggesting that this transcription factor is a relevant target for potential anti-inflammatory agents (Baldwin, 1996).

In most cells, NF-κB exists as an inactive heterodimer, the predominant form of which is composed of p50 and p65 (RelA) subunits (Fujita *et al.*, 1993). Both the p50 and p65 monomers contain Rel regions approximately 300 amino acids in length, that bind to DNA, interact with each other, and bind to IκB (Baldwin, 1996; Zabel and Baeuerle, 1990). In the cytosol, NF-κB sequestered as an inactive complex with an inhibitory molecule of the IκB family (Baeuerle and Baltimore, 1988; Baldwin, 1996; Karin and Smeal, 1992). All six known members of the IκB family (IκBα, IκBβ, IκBγ, Bcl-3, p100, and p105) (Beg *et al.*, 1992; Fujita *et al.*, 1993) contain an ankyrin (ANK) repeat domain that consists of five to seven closely adjacent repeats required for both association with NF-κB and the inhibitory activity (Baeuerle and Baltimore, 1996).

Recently, considerable attention has been focussed on identifying naturally occurring chemopreventive substances capable of inhibiting, retarding, or reversing the multi-stage carcinogenesis. A wide array of phenolic substances, particularly those present in dietary and medicinal plants, have been reported to possess substantial anticarcinogenic and antimutagenic activities (recently reviewed by Surh, 1999 and references therein). The majority of naturally occurring phenolics retain antioxidative and anti-inflammatory properties which appear to contribute to their chemopreventive or chemoprotective activity. Since inflammation and oxidative tissue damage are closely linked to tumor promotion, substances with potent anti-inflammatory and/or antioxidative activies are anticipated to exert chemopreventive effects on carcinogenesis, particularly in the promotion stage. An example is curcumin, a yellow pigment of turmeric (*Curcuma longa* L., Zingiberaceae), that strongly suppresses tumor promotion. Recent studies from this laboratory have demonstrated that some naturally occurring diaryl hepatanoids structurally related to curcumin have substantial anti-tumor promotional activities. Thus, yakuchinone A [1-(4'-hydroxy-3'-methoxyphenyl)-7-phenyl-3-heptanone] and yakuchinone B [1-(4'-hydroxy-3'-methoxyphenyl)-7-phenylhept-1-en-3-one] present in *Alpinia oxyphylla* Miquel (Zingiberacease) attenuate phorbol ester-induced inflammation, production of reactive oxygen species and skin tumor promotion in mice. These diarylheptanoids (structures shown in Figure 2) suppress phorbol ester-induced activation of ornithine decarboxylase and its mRNA expression in mouse skin (Chun *et al.*, 1999). Likewise, phorbol ester-induced enhancement of epidermal TNF-α production and its mRNA expression was mitigated by pretreatment with yakuchione A or B. Moreover, both compounds inhibit phorbol ester-induced expression of COX-2 and iNOS in mouse skin, possibly via inactivation of NF-κB. Capsaicin, a major pungent ingredient of red pepper also attenuated phorbol ester-induced NF-κB activation (Surh *et al.*, 2000). Similar suppression of COX-2 and iNOS and down-regulation of NF-κB activation for its DNA binding were observed with the ginsenoside Rg_3 and the ethanol extract of *Artemisia asiatica.* We have also found that certain anti-inflammatory phytochemicals exert inhibitory effects on phorbol ester-induced COX-2 expression and NF-κB activation in immortalized human breast epithelial (MCF-10A) and human promyelocytic leukemia (HL-60) cells in culture. One of the plausible mechanisms underlying inhibition by aforementioned phytochemicals of phorbol ester-induced NF-κB activation involves interference with degradation of the inhibitory unit, I-κB and subsequent nuclear translocation of the functionally active p65 subunit of NF-κB. The activation of epidermal NF-κB by phorbol ester and subsequent induction of COX-2 hence appears to play an important role in intracellular signaling pathways (Figure 3) leading to tumor promotion and targeted inhibition of NF-κB may provide a new promising cancer chemopreventive strategy.

References

Baeuerle, P.A. and Baltimore, D. (1988) Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. *Cell*, 53, 211-217.

Baeuerle, P.A. and Baltimore, D. (1996) NF-κB: 10 years after. *Cell*, 87, 13-20.

Baldwin, A.S. (1996) The NF-kB and I-kB proteins: new discoveries and insights. *Annu. Rrev. Immunol.*, 14, 649-681.

Beg, A.A., Ruben, S.M., Scheinman, R.I., Haskill, S., Rosen, C.A., and Baldwin, A.S.Jr. (1992) I-κB interacts with the nuclear localization sequences of the subunits of NF-kB: a mechanism for cytoplasmic retention. *Genes Dev.*, 6, 1899-1913.

Blank, V., Kourilsky, P., and Israel, A. (1992) NF-κB and related proteins: Rel/dorsal homologies meet ankyrin-like repeats. *Trends Biochem. Sci.*, 17, 135-140.

Chun, K.-S., Sohn, Y., Kim, H.-S., Kim, O.H., Park, K.-K., Lee, J.-M., Lee, J., Lee, J.Y., Moon, A., Lee, S.S., and Surh, Y.-J. (1999) Anti-tumor promoting potential of naturally occurring diarylheptanoids structurally related to curcumin. *Mutat. Res.*, 428, 49-57.

Cobbs, C.S., Brenman, J.E., Aldape, K.D., Bredt, D.S., and Israel, M.A. (1995) Expression of nitric oxide synthase in human central nervous system tumors. *Cancer Res.*, 55, 727-730.

Crofford ,L.J., Wilder, R.L., Ristimaki, A.P., Sano, H., Remmers, E.I., Epps, H.R., and Hla, T. (1994) Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissue. Effects of interleukin-1a, phorbol ester, and corticosteroid. *J. Clin. Invest.*, 93, 1095-1101.

Dubois, R.N., Abramson, S.B., Crofford ,L., Grupta, R.A., Simon, L.S., Vaade Putte, L.B.A., and Lipsky, P.E. (1998) Cyclooxygenase in biology and disease. *FASEB J.*, 12, 1063-1073.

Earnest, D.V., Hixson, L.J., and Alberts, D.S. (1992) Piroxicam and other cyclooxygenase inhibitors: potential for cancer chemoprevention. *J. Cell. Biochem.*, 161 (suppl), 156-166.

Fujita, T., Nolan, G.P., Liou, H.C., Scott, M.L., and Baltimore, D. (1993) The candidate protooncogene bcl-3 encodes a transcriptional coactivator that activates through NF-κB p50 homodimers. *Genes Dev.*, 7, 1354-1363.

Gallo, O., Masini, E., Morbidelli, L., Franchi, A., Fini-Storchi, I., Vergari, W.A., and Ziche, M. (1998) Role of nitric oxide in angiogenesis and tumor progression in head and neck cancer. *J. Natl. Cancer. Inst.*, 90, 587-596.

Grilli, M., Chiu, J.J.-S., and Lenardo, M.J. (1993) NF-kB and rel-participants in a multiform transcriptional regulatory system. *Int. Rev. Cytol.*, 143, 1-62.

Hla, T., Ristimaki, A. Appleby, S., and Barriocanal, J.G. (1993) Cyclooxygenase gene expression in inflammation and angiogenesis. *Ann. N. Y. Acad. Sci.*, 696, 197-204.

Inoue, H., Yokoyama, C., Hara, S., Tone, Y., and Tanabe, T. (1995) Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phobol ester in vascular endothelial cells. *J. Biol. Chem.*, 270, 24965-24971.

Kargman, S.L., O'Neil, G.P., Vickers, P.J., Evans, J.F., Mancini, J.A., and Jothy, S. (1995) Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. *Cancer Res.*, 55, 2556-2559.

Karin, M. and Smeal, T. (1992) Control of transcription factors by signal transduction pathways: the beginning of the end. *Trends Biochem. Sci.*, 17, 418-422.

Kawamori, T., Rao, C.V., Seibert, K., and Reddy, B.S. (1998) Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res.*, 58, 409-412.

Kopp, E.B. and Ghosh, S. (1995) NF-κB and rel proteins in innate immunity. *Adv. Immunol.*, 58, 1-27.

Kutchera, W., Jones, D.A., Matsunami, N., Groden, J., McIntyre, T.M., Zimmerman, G.A., White, R.N., and Prescott, S.M. (1996) Prostaglandin H synthase 2 is expressed abnormally in human colon cancer: evidence for a transcriptional effect. *Proc. Natl. Acad. Sci. USA.*, 93, 4816-4820.

Marnett, L.J (1992) Aspirin and the potential role of prostaglandins in colon cancer. *Cancer Res.*, 52, 5575-5589.

Mordan, L.J., Burnett, T.S., Zhang, L.X., Tom, J., and Cooney, R.V. (1993) Inhibitors of endogenous nitrogen oxide formation block the promotion of neoplastic transformation in C3H 10T1/2 fibroblasts. *Carcinogenesis*, 14, 1555-1559.

Muller-Decker, K., Scholz, K., Marks, R., and Furstenberger, G. (1995) Differential expression of prostaglandin H synthase isozymes during multistage carcinogenesis in mouse epidermis. *Mol. Carcinog.*, 12, 31-41.

Ohshima, H. and Bartsch, H. (1994) Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat. Res.*, 305, 253-264

Oshima, M., Dinchuk, J.E., Kargman, S.L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J.M., Evans, J.F., and Taketo, M.M. (1996) Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell*, 87, 803-809.

Radomski, W., Jenkins, D.C., Holmes, L., and Moncada, S. (1991) Human colorectal adenocarncinoma cells: differential nitric oxide synthesis determines their ability by to aggregate platelets. *Cancer Res.*, 51, 6073-6078.

Rigas, B., Goldman, I.S., and Levine, L. (1993) Altered eicosanoid levels in human colon cancer. *J. Lab. Clin. Med.*, 122, 518-523.

Ristimaki, A., Honkanen, N., Jankala, H., Sipponen, P., and Harkonen, M. (1997) Expression of cyclooxygenase-2 in human gastric carcinoma. *Cancer Res.*, 57, 1276-1280.

Rosbe, K.W., Prazma, J., Petrusz, P., Mims, W., Ball, S.S., and Weissler, M.C. (1995) Immunohistochemical characterization of nitric oxide synthase activity in squamous cell carcinoma of the head and neck. *Otolaryngol Head Neck Surg.*, 113, 541-9.

Schmitz, M.L. and Baeuerle, P.A. (1995) Multi-step activation of NF-kappa B/Rel transcription factors. *Immunobiol.*, 193, 116-127.

Sheng, G.G., Shao, J., Sheng, H., Hooton, E.B., Isakson, P.C., Morrow, J.D., Coffey, R.J., Dubois, R.N., and Beauchamp, R.D. (1997) A selective cyclooxygenase 2 inhibitor suppresses the growth of H-*ras*-transformed rat intestinal epithelial cells. *Gastroenterology*, 113, 1883-1891.

Subbaramaiah, K., Telang, N., Ramonetti, J.T., Araki, R., Devito, B., Weksler, B.B., and Dannenberg, A.J. (1996) Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. *Cancer Res.*, 56, 4424-4429

Surh, Y.-J. (1999) Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. *Mutat. Res.*, 428, 305-327.

Surh, Y.-J., Han, S.S., Seo, H.-J., and Lee, S.S. (2000) Inhibitory effects of curcumin and capsaicin on phorbol ester-induced activation of eukaryotic transcription factors, NF-κB and AP-1. *BioFactors*, 12, in press.

Szabo, C. (1995) Alterations in nitric oxide production in various forms of circulatory shock. *New Horiz.*, 3, 2-32.

Thomsen, L.L., Lawton, F.G., Knowles, R.G., and Beesley, J.E. (1994) Nitric oxide synthase activity in human gynecological cancer. *Cancer Res.*, 54, 1352-1354.

Thomsen, L.L., Miles, D.W., Happerfield, L., Bobrow, L.G., Knowles, R.G., and Moncada, S. (1995) Nitric oxide synthase activity in human brest cancer. *Br. J. Cancer*, 72, 41-44.

Tsujii M., Kawano, S., and DuBois, R.N. (1997) Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc. Natl. Acad. Sci. USA*, 94, 3336-3340.

Yun, H.Y., Dawson, V.L. and Dawson, T.M. (1996) Neurobiology of nitric oxide. *Crit. Rev. Neurobiol.*, 10, 291-316.

Zabel, U. and Baeuerle, P.A. (1990) Purified human I-κB can rapidly dissociate the complex of the NF-κB transcript on factor with its cognate DNA. *Cell*, 61, 255-265.

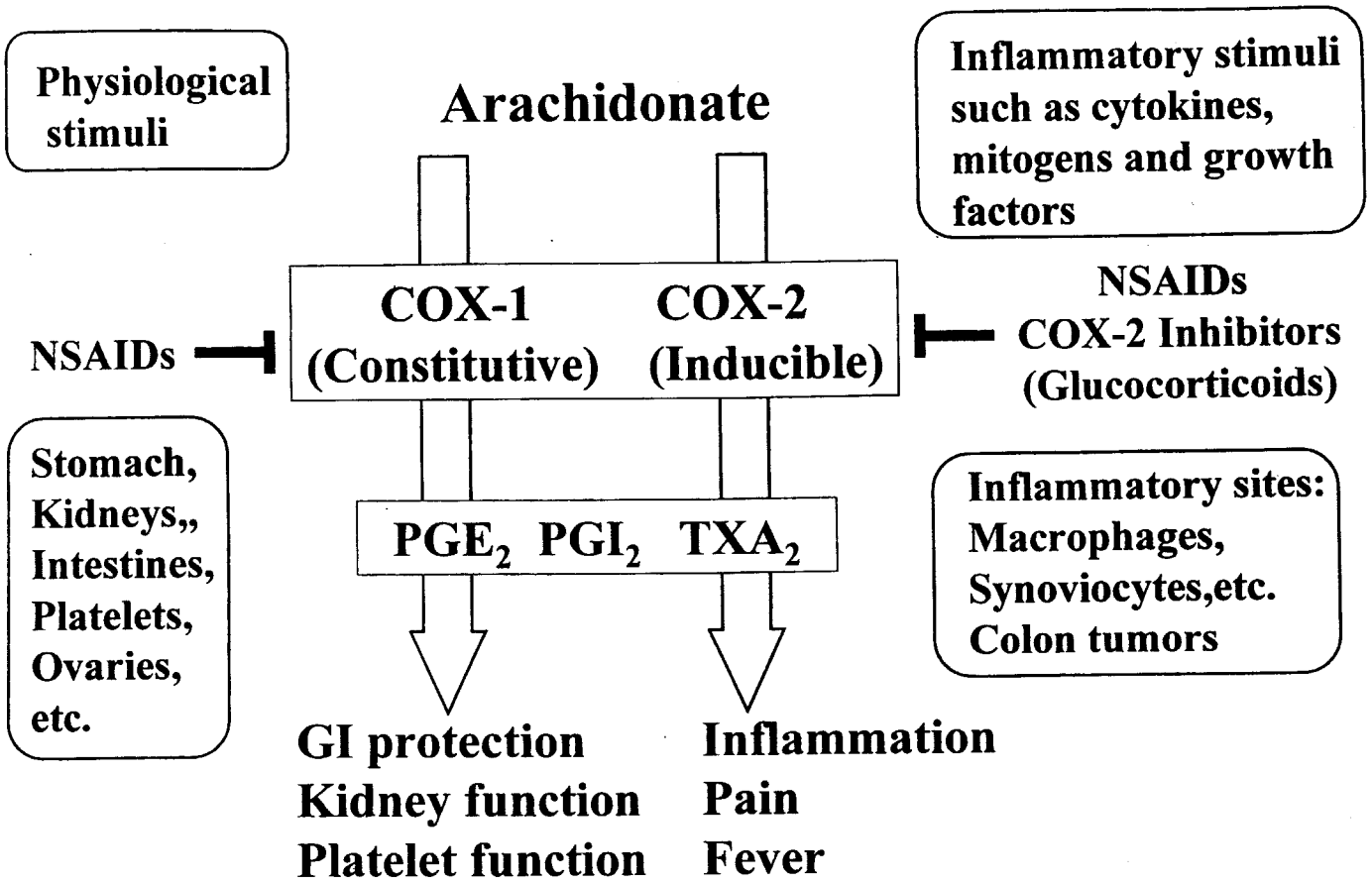

Figure 1. Cyclooxygenase (COX) pathways.

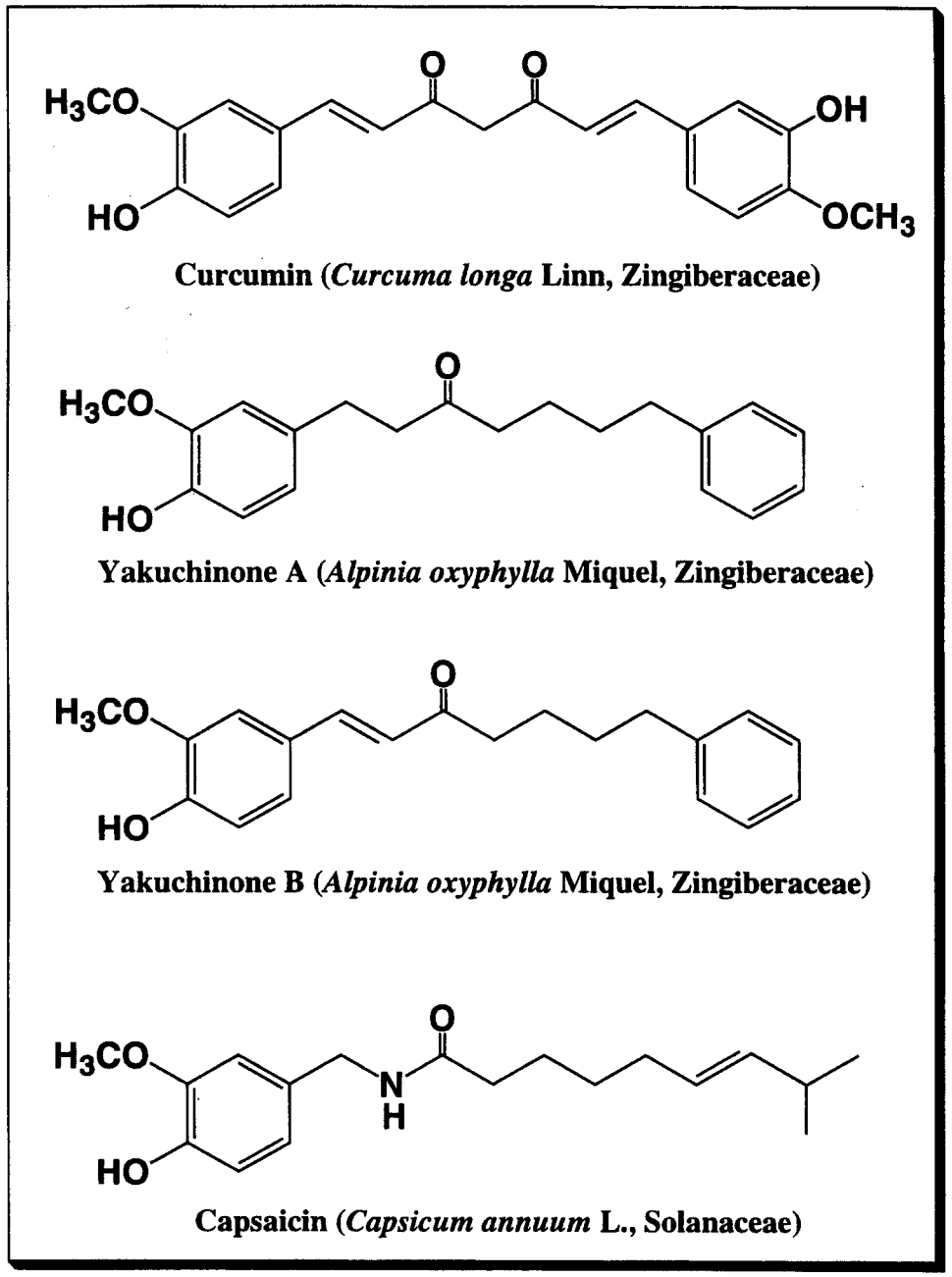

Figure 2. Chemical structures of curcumin, yakuchinone A & B, and capsaicin

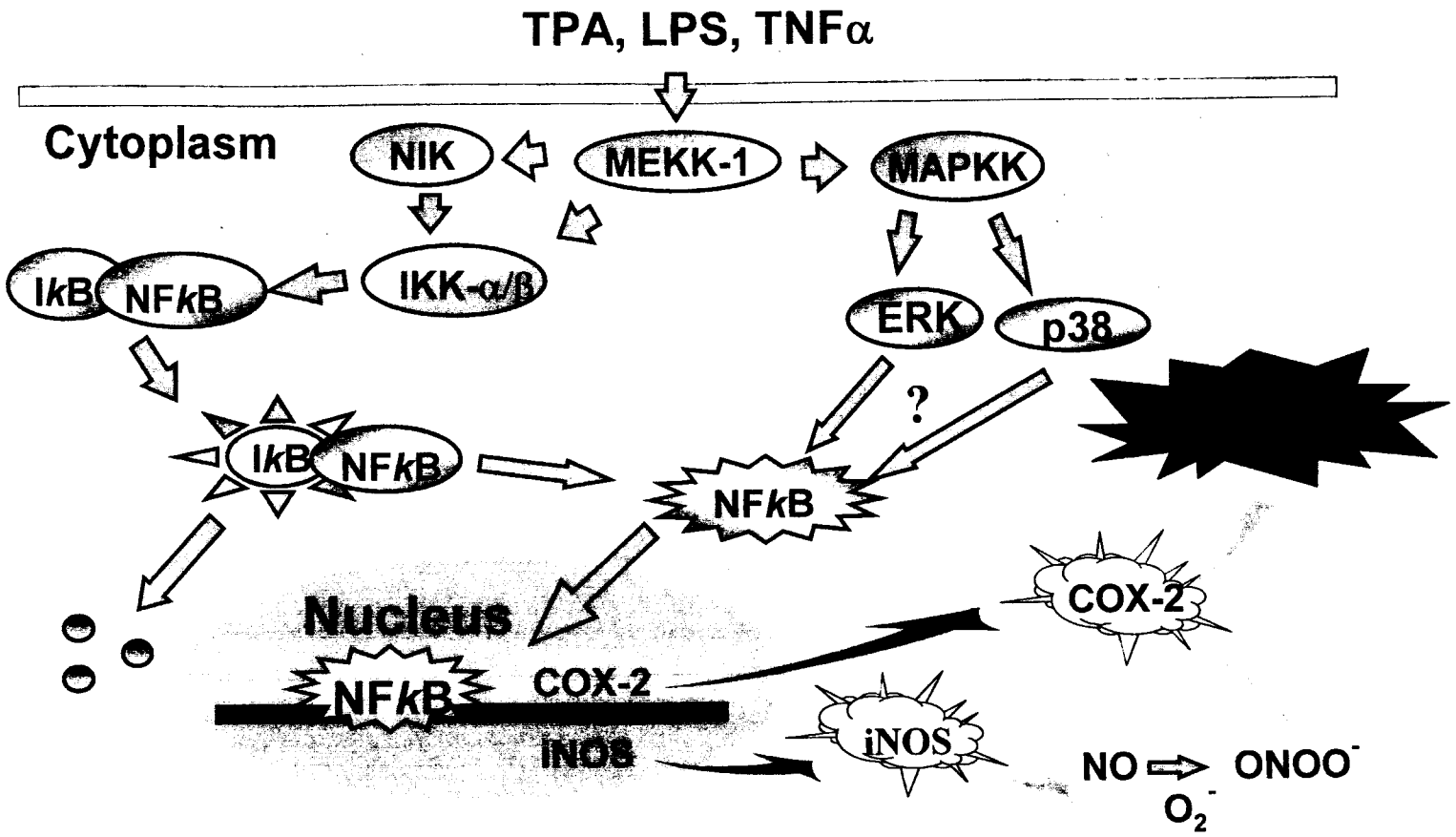

Figure 3. Schematic illustration of proposed intracellular signaling pathways leading to induction of COX-2 and iNOS expression.