Phospholipase D isozymes mediate epigallocatechin gallate-induced cyclooxygenase-2 expression in astrocyte cells

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Summary

Little is known about the effect of epigallocatechin-3 gallate (EGCG), a major constituent of green tea, on the expression of cyclooxygenase (COX)-2. Here, we studied the role of phospholipase D (PLD) isozymes in EGCG-induced COX-2 expression. Stimulation of human astrocytoma cells (U87) with EGCG induced formation of phosphatidylbutanol, a specific product of PLD activity, and synthesis of COX-2 protein and its product, prostaglandin E_2 (PGE_2). Pretreatment of cells with 1-butanol, but not 3-butanol, suppressed EGCG-induced COX-2 expression and PGE_2 synthesis. Furthermore, evidence that PLD was involved in EGCG-induced COX-2 expression was provided by the observations that COX-2 expression was stimulated by over-expression of PLD1 or PLD2 isozymes and treatment with phosphatidic acid (PA), and that prevention of PA dephosphorylation by 1-propranolol significantly potentiated COX-2 expression induced by EGCG. EGCG induced activation of p38 mitogen-activated protein kinase (p38 MAPK), and specific inhibition of p38 MAPK dramatically abolished EGCG-induced PLD activation, COX-2 expression, and PGE_2 formation. Moreover, protein kinase C (PKC) inhibition suppressed EGCG-induced p38 MAPK activation, COX-2 expression, and PGE_2 accumulation. The same pathways as those obtained in the astrocytoma cells were active in primary rat astrocytes, suggesting the relevance of the findings. Collectively, our results demonstrate for the first time that PLD isozymes mediate EGCG-induced COX-2 expression through PKC and p38 in immortalized astroglial line and normal astrocyte cells.

Key words: PLD, COX-2, PGE_2, EGCG, PKC, p38 MAPK

Cyclooxygenase (COX) is the key enzyme in themetabolic pathway leading to prostaglandin (PG) and thromboxane A2 formation from arachidonic acid. Two isoforms have been identified, COX-1 and COX-2. COX-1 is constitutively expressed in nearly all normal mammalian tissues, and mediates the synthesis of PGs required for physiological tissue homeostasis. In contrast, COX-2 expression is rapidly induced in response to various stimuli, including inflammatory signals, mitogens, cytokines, and growth factors in a wide variety of cells such as macrophages, microglia, and astrocytes. COX-2 has also been in normal brain, in discrete populations of neurons. The function of basal prostanoid production in brain is unclear. Green tea polyphenols, which comprise 30% of the dry weight of green tea leaves, include epigallocatechin-3-gallate (EGCG), epigallocatechin, epicatechin-3-gallate, and epicatechin. EGCG is the most abundant of these catechins and it has been attributed many healthful benefits.

However, a recent study demonstrates that EGCG up-regulates COX-2 expression and prostaglandin

E_2 (PGE_2) production in Raw 264.7 macrophage cells, suggesting that EGCG may enhance inflammatory processes. However, the downstream effectors linking EGCG stimulation with COX-2 expression and PG production remains unidentified, although the effects of EGCG on COX-2 expression still remain uncertain.

Recently, several studies have implicated a PLD-derived signaling pathway in the generation of prostaglandinin many cell types. PLD catalyzes the hydrolysis of phosphatidylcholine to generate a lipid mediator, phosphatidic acid (PA). In mammals, two isoforms of PLD, PLD1 and PLD2, have been cloned and are being characterized for regulation and cellular function. However, segregated roles of the two PLD isoforms in cellular responses are still poorly understood. Several lines of evidence have suggested a functional role for PLD in COX-2 regulation during cell activation. However, the role of PLD isozymes in EGCG-induced COX-2 expression has not been studied in any biological system. We therefore investigated a role of PLD in the regulation of COX-2 expression in glial cells treated with EGCG. In this study, we demonstrate that EGCG activates PLD through an upstream protein kinase C to elicit p38 activation and finally induce COX-2 expression in normal rat astrocyte cells and glioma cells. This is the first study to show the involvement of PLD isozymes in mediating EGCG-induced COX-2 expression in any biological system.

Green tea is the most popular and widely consumed beverage in the world, after water, and is reported to possess beneficial health effects for humans. These beneficial health effects have attracted considerable diseases, including cancer and cardiovascular and neurodegenerative diseases. Studies have verified that polyphenols in green tea are potent antioxidants, with majority of beneficial effects elicited by EGCG, one of the main constituents of green tea. However, the present study demonstrates that EGCG upregulates COX-2 expression and PGE_2 production in astroglioma cells.

The major finding of this study is that PLD is a vital component of the signal transduction pathway induced by EGCG that leads to the expression of COX-2 in human astroglioma cells. To the best of our knowledge, this is the first study to link PLD isozymes to EGCG-induced COX-2 expression in any cell system.

Some reports have described an inhibitory effect of EGCG on COX-2 upregulation induced by agonists such as PMA, N-nitrosomethylbenzylamine, or interleukin-1? (IL-1?). However, in human chondrocytes, treatment of EGCG alone showed a moderate increase in COX-2 expression (approximately 4-fold relative to control) and COX-2 activity (PGE_2 formation) when compared to the untreated control. In addition, Park *et al.* have reported that in the macrophage cell line, Raw 264.7, COX-2 expression and PGE_2production are increased by EGCG treatment. Therefore, it seems that the effects of EGCG on COX-2 expression and PGE_2 production still remain uncertain.

Little is known about how COX-2 protein levels are regulated in glial cells, the PLDs responsible for such a regulation, and the molecular mechanisms involved.

We have studied signal transduction pathways involved in EGCG-induced COX-2 expression in U87 MG human astroglioma cells. EGCG stimulated COX-2 protein expression and PGE_2 production as well as PLD activity.

Some studies have implicated a PLD-derived signaling pathway in the generation of prostaglandins in many cell types. However, PLD-independent signaling pathways must exist for the production of prostaglandins since EGF, which has been shown to induce COX-2, was demonstratednot to activate PLD in the studies by Sciorra and colleagues. Kaneki *et al.* showed that PMA-induced COX-2 expression in osteoblast-like UMR-106 cells was dependent upon PLD activity.As a control for non-specific effects of butanol, the secondary and tertiary forms of butanol have often been employed. The evidence identifying PLD activity as important in functional events relies on the use of alcohols. Importantly, inhibition of PA formation through the addition of the primary alcohol 1-butanol blocked

EGCG-induced COX-2 expression, whereas 3-butanol, which does not participate in transphosphatidylation, did not. Furthermore, direct additionof PA to the cells was very effective in stimulating COX-2 expression. Interestingly, propranolol, an inhibitor of PA phosphatase, strongly potentiated EGCG-induced COX-2 expression. These results demonstrate that PLD activity and the intracellular accumulation of PA are importantly involved in EGCG-induced COX-2 expression.

It is well known that PKC acts as a mediator of a broad spectrum of effects, including the activation of PLD. Activation of PKC has been suggested to be a key event in the signaling pathway leading to COX-2 expression. In the present study, we found that EGCG-induced PLD activation and COX-2 expression were reduced by a PKC inhibitor, indicating that PKC activation is involved in the signal transduction leading to PLD activation and COX-2 expression by EGCG.

Recently, it has been reported that COX-2 expression can be regulated through different MAP kinase signaling pathways and that the particular signaling pathway involved is dependent on the type of stimuli. In U87 astroglioma cells,EGCG induced phosphorylation of ERK and p38 MAP kinase. The observed increase in phosphorylated ERK levels by EGCG was transient with peak enhancement of phosphorylation being evident at 10 min post-stimulation. By contrast, p38 MAPK exhibited sustained activation, with a maximum at 30 min post-stimulation, which was still evident 50 min after EGCG treatment. We used MAPK inhibitors to examine whether MAPK activation was involved in the signal transduction pathway leading to COX-2 expression caused by EGCG.. SB203580, a selective inhibitor of p38, strongly suppressed EGCG-induced COX-2 expression, but PD98059, a selective inhibitor of MEK, did not.

Our results suggest that EGCG may act on two pathways to enhance COX-2 synthesis in U87 cells, i.e., via activation of PKC and p38 MAPK. Very recently, Kim *et al.* demonstrated that sphingosine-1-phosphate (S1P) in amniotic fluid modulated COX-2 expression via ERK, but not p38 kinase in human amnion-derived WISH cells. In addition, S1P-induced COX-2 expression wasnot affected in the presence of 1-butanol. It was reported that induction of COX-2 by interleukin-1 (IL-1) is mediated by both ERK and p38 kinase in murine astrocytes. These results suggest that the intracellular signaling pathways involved in the modulation of COX-2 by S1P or IL-1 differ from those involved in the modulation of COX-2 by EGCG.. Fiebich *et al.* demonstrated a similar role for PKC and p38 MAPK activation of COX-2 in SH-N-SK human neuroblastoma cells.

However, there is no direct evidence todate that indicates direct regulation of COX-2 expression by PLD protein levels. Our current observation that over-expression of PLD1 or PLD2, caused by ectopic expression in cells, leads to increased expression of COX-2, clearly indicates a positive rolefor PLD isozymes in COX-2 expression in human U87 MG astroglioma cells. To the best of knowledge, this is the first direct indication that suggests regulation of COX-2 expression by PLD protein levels. We and other groups have reported the link between p38 and PLD activity. The idea that both PLD1 and PLD2 can couple to regulation of COX-2 expression by EGCG is an interesting one that might fit with the findings that both PKC and p38 are involved. COX-2 expression in PLD1 expressing cells was more sensitive to PKC inhibition than that in PLD2 expressing cells. It might be due to more responsiveness of PLD1 to PKC. We further used cultured primary rat astroglial and microglial cells to corroborate our findings. EGCG induced COX-2 gene expression in primary rat astrocytes and microglial cells, in line with the increase observed in human astroglioma cells. Moreover, we found that PLD mediated EGCG-induced COX-2 expression through PKC and p38 in normal astrocyte cells. Thus, the same pathways as those obtained in the astrocytoma cells were active in primary rat astrocytes, suggesting the relevance of the findings.

COX-2 expression is regulated in not only a cell type-specific manner but also a species-specific manner. Induction of COX-2 expression may represent anovel mechanism by which EGCG mediate its diverse actions within the central nervous system.

The conclusion that EGCG mediates COX-2 expression may seem surprising or even paradoxical, because the actions of EGCG and COX-2 are generally thought to be beneficial and detrimental, respectively. Specifically, induction of COX-2 is believed to play a role in inflammation, toxic shock, cancer, and apoptosis. However, there is evidence suggesting physiologically important or salutary actions of COX-2 in other situations. For example, COX-2 protects cardiomyocytes against oxidative stress, and exerts anti-apoptotic actions in various cell types. The finding that genetic disruption of COX-2 results in cardiac fibrosis also suggests that COX-2 expression may be protective. Interestingly, the clinical experience accumulated with COX-2 inhibitors suggests that COX-2 exerts protective effects in patients with cardiovascular disease. A concept is emerging that COX-2 induction represents an important compensatory mechanism to defend against vascular injury. Furthermore, it is now recognized that COX-2 is constitutively expressed in the kidney and the brain, and plays an important role in maintaining renal function and in modulating neural responses. Recently, it was proposed that the pathophysiological roles of COX-2 are much more complex than hitherto appreciated, and that this enzyme may exert either beneficial or deleterious effects depending on the intensity of its induction, the pathophysiological setting, and the ability of specific cells to metabolize PGH_2,produced by COX-2, into cytoprotective prostanoids. The pathological role that COX-2 plays may depend on a number of factors, among which the cell types and their inherent prostanoid synthetic pathways appear to bethe key determinants. It was suggested that throughout adult life, COX-2 might remain an important modulator of specific neural responses. EGCG has been demonstrated to pass the blood-brain barrier and reach the brain parenchyma in animal studies, and detection of EGCG in rat brain suggests polyphenols can modulate neuronal activity. COX-2 also seems to play an essential role in neural development and adaptation. At present, the role of COX-2 in human brain function and the potential impact of specific COX-2 inhibitors are unknown and requires evaluation, especially in view of the well-known negative impact of non-specific COX inhibitors on cognitive function in the elderly. To the best of our knowledge, this is the first report to show that EGCG might activate PLD through an upstream protein kinase C to elicit p38 activation and finally induce COX-2 expression.

In summary, the involvement of PLD1 and PLD2 isozymes in EGCG-induced COX-2 expression has been explored. Since this study is the only report upon the potential role of EGCG in glial cells, further studies on the physiological roles of EGCG are required and necessary to determine overall signal transduction pathways that are associated with EGCG-induced COX-2 regulation..

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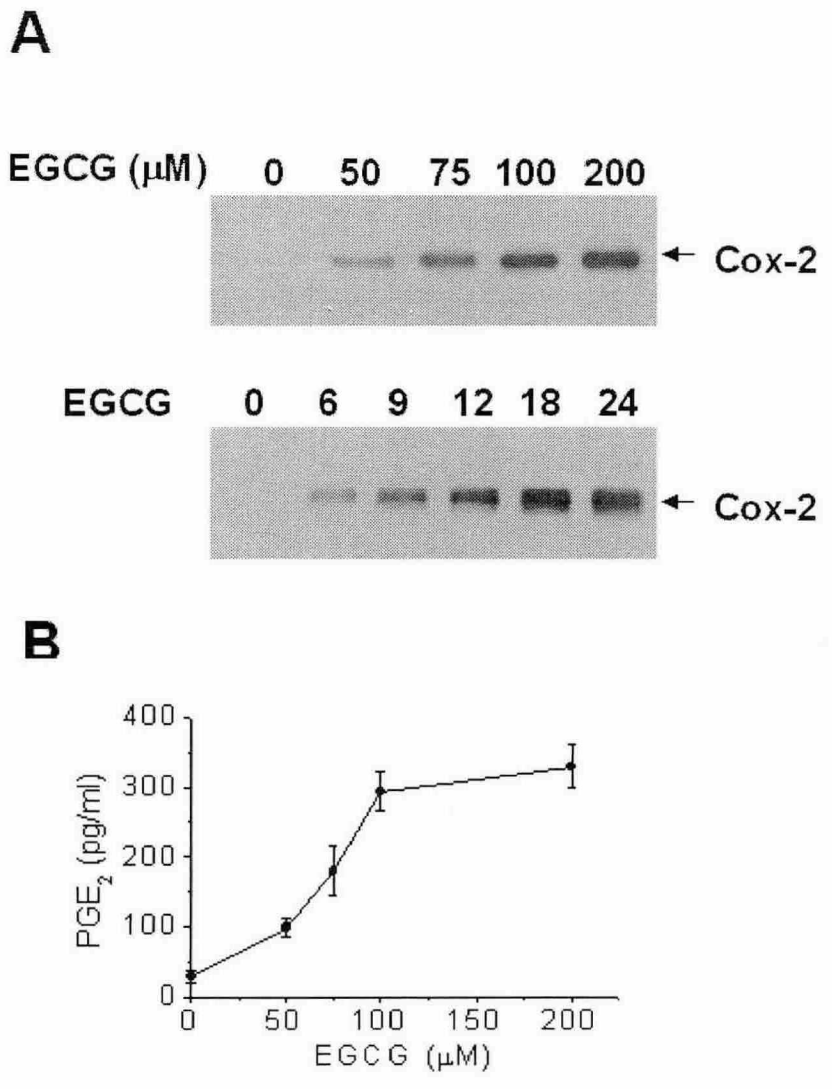

FIG. 1. **Effect of EGCG on COX-2 expression and PGE_2 accumulation.** A) U87 cells were treated with or without various concentrations of EGCG for 12 h or 100 ?M EGCG for the times indicated. Cell lysates were prepared and analyzed for COX-2 expression by Western blot analysis, using specific antibodies as described in "Experimental Procedures". These blots are representative of results obtained from three experiments. B) Cells were stimulated with the indicated concentrations of EGCG for 6 h, and then the release of PGE_2 was measured from supernatants as described in "Experimental Procedures". The values shown for PGE2 production are the mean ± S.E. of three independent experiments.

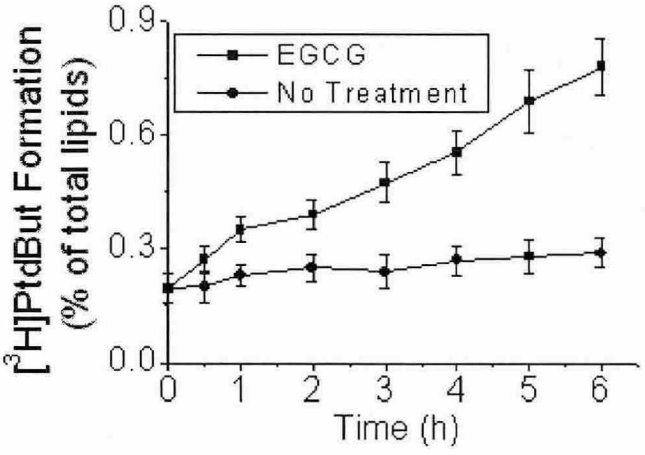

FIG. 2. **Activation of PLD in EGCG-treated U87 cells.** Cells were serum-deprived and labeled with 2 ?Ci/ml [^{3}H] myristic acid. Following washing and preincubation with DMEM, 0.1% BSA, 0.3% 1-butanol for 20 min, 100 ?M EGCG was added, and cells were incubated for the indicated times at 37 ℃. Lipids were extracted and analyzed, and the measurement of [^{3}H] PtdBut production was as described in "Experimental Procedures". [^{3}H] PtdBut values were normalized by dividing the measured counts/min by the counts/min in the total lipid fraction. Data are expressed as the mean of the means ± S.E. of three independent experiments.