T-614, a Novel Antirheumatic Drug, Inhibits Both the Activity and Induction of Cyclooxygenase-2 (COX-2) in Cultured Fibroblasts

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ABSTRACT—To elucidate the mechanism for the selective inhibition of prostaglandin E₂ (PGE₂) production in inflammatory tissue by T-614 (3-formylamino-7-methylsulfonylamino-6-phenoxy-4H-1-benzopyran-4-one), its effects on both the activity and the induction of cyclooxygenase (COX)-2 were investigated in vitro. T-614 inhibited the activity of purified COX-2 enzyme (IC₅₀: 7.7 µg/ml), but was inactive against both COX-1 activities of microsomal and purified enzymes (IC₅₀: > 300 µg/ml). On the other hand, when the inhibition of PGE₂ production by T-614 was examined in the cultured fibroblasts stimulated with bradykinin, T-614 at 1 µg/ml or less inhibited PGE₂ release more effectively than that in the above cell-free system. Therefore, we examined which of the COX enzymes was expressed in bradykinin-stimulated fibroblasts by using both the reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analyses. As a result, COX-1 mRNA was constitutively expressed in the cells, whereas COX-2 mRNA was not detected without stimulation with bradykinin, but was expressed within 30 min when stimulated. Furthermore, it was found that the addition of T-614 reduced the COX-2 mRNA levels in 30 min after stimulation. These studies suggest that at least some of inhibitory effects of T-614 on prostanoids production are mediated by the synergy of the inhibition of COX-2 activity and the inhibition of induction, and such an action of T-614 may explain the pharmacological properties of this drug.

Keywords: T-614, Cyclooxygenase-2, Prostaglandin production, Bradykinin, Reverse transcriptase-polymerase chain reaction

Cyclooxygenase (COX) is a rate-limiting enzyme in prostanoids (PGs) biosynthesis (1). The 2.8-kilobase complementary DNA (cDNA) of COX was cloned in mammalian cells (2, 3). Recently, a mitogen-inducible form of COX encoded by a 4.1-kilobase mRNA has been discovered in mice (4), humans (5) and chickens (6). This isoform is designated as COX-2, and the first discovered form is designated as COX-1. The two isoforms show about 60% homology in both their nucleic acid and amino acid structures. In contrast, COX-1 and COX-2 are pharmacologically distinct. For instance, although COX-1 is a constitutively expressed enzyme that is present in most tissues (7), COX-2 is an inducible enzyme present especially in inflammatory tissues (8, 9) and mitogen-stimulated cells (4).

Non-steroidal antiinflammatory drugs (NSAIDs) are generally believe to exert their antiinflammatory effect through inhibition of COX activity (10). We have already reported that a novel antirheumatic drug, 3-formylamino-7-methylsulfonylamino-6-phenoxy-4H-1-benzopyran-4-one (T-614), reduced the prostaglandin E₂ (PGE₂) contents in the inflammatory exudate of rats in vivo, but not in the gastric mucosa (11). In addition, COX activity of rabbit renal microsomes, a crude COX enzyme, was very weakly inhibited by T-614, but T-614 effectively inhibited the PGE₂ production by mouse fibroblasts stimulated with bradykinin. Therefore, we have suggested that this drug inhibits PGs production by an apparently different mode from conventional NSAIDs. Such inhibition by T-614 may be restricted to inflammatory tissues and to the related cells activated with a certain stimulus (11).

Recent reports that COX-2 is induced in cells exposed to proinflammatory agents, such as cytokines and endotoxin (12, 13), and that it is responsible for the production of PGs at the site of inflammation (14) make it possible for us to deduce the direct and specific effects of T-614 on COX-2. Inhibition of COX-2 activity by T-614 may be achieved at several steps in the cascade of events leading to induction of the enzyme activity. In this report, we examined the effect of T-614 on the activities of standard en-
zyme preparations of COX-1 and COX-2 in cell-free system. Moreover, it was confirmed that COX-2 was induced at the mRNA level in fibroblasts stimulated with bradykinin by using both the reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analyses, and the effect of T-614 on the COX-2 induction was examined.

MATERIALS AND METHODS

Cells

Mouse 3T3 fibroblast cells (ATCC, CCL 163) were maintained in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo) with 10% fetal calf serum (FCS; Gibco BRL, Grand Island, NY, USA). The cells were cultured at 37°C in a humidified atmosphere of 5% CO2/95% air.

Drugs

T-614 synthesized by Toyama Chemical Co. and indomethacin (Sigma Chemical Co., St. Louis, MO, USA) were used. These drugs were dissolved in dimethylsulfoxide (DMSO) and added to the reaction buffer or medium at the indicated concentration (final concentration of DMSO: 1% or less). All the chemicals used were of reagent grade.

Effect on standard enzyme preparations of COX-1 and COX-2

Sheep seminal vesicle microsomes enriched in constitutive COX activity, as a COX-1 preparation (15), were obtained from Edoman Technologies (Jerusalem, Israel). As the purified enzyme preparations, COX-1 isolated from ram seminal vesicles (purity >95%) and COX-2 isolated from sheep placenta (purity 70%) were from Cayman Chemical (Ann Arbor, MI, USA). Enzyme activity was measured by the conversion of labeled arachidonate to PGs after the selective extraction method, as described by Yanagi and Komatsu (16). A solution (5 µl) of T-614 or indomethacin was added to an assay mixture containing 100 µg/ml COX-1 microsomes or 10 units/ml purified COXs, which was then made up to a final volume of 0.5 ml with 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM glutathione and 5 mM epinephrine. In the assay of the purified enzymes, 1 µM hematin was added to the mixture as an additional cofactor. The reaction mixture was preincubated for 2 min at 37°C. [1-14C]Arachidonic acid [10 nmol for microsomes or 3.3 nmole (17) for purified COXs; 88,000 dpm, Amersham Lab., Buckinghamshire, UK] was then added as the substrate, and the mixture was further incubated for 4 min. After adding 2 ml of n-hexane : ethyl acetate (2:1, v/v) and centrifuging the preparation, the organic solvent phase was removed to eliminate any remaining arachidonic acid. The extraction procedure was repeated 3 times. Since PGs selectively remained in the aqueous phase, radioactivity of the aqueous phase was measured as a PGs fraction by using a liquid scintillation counter. An aliquot of the total organic phase (about 6 ml) was sampled to measure the radioactivity as an unchanged arachidonic acid fraction. The conversion rate of arachidonic acid to PGs was calculated from the determinations of both fractions. Under these conditions, PGE2 was the main product in all the COX preparations, and more than 95% of the radioactivity in the reaction mixture was recovered.

Stimulation of fibroblasts with bradykinin

The fibroblasts were stimulated with bradykinin according to the previously reported method (11), with some modifications. In brief, the cells were passaged into 100-mm culture dishes at a cell density of 5 x 106 cells per dish. After 24-hr culture, the medium was removed from the dish, and fresh FCS-free medium was added together with T-614, indomethacin or the solvent alone (DMSO). After a 15-min preincubation, bradykinin (1 µM) was added, and the cells were incubated for the times indicated at 37°C. Then, the conditioned media were removed to measure the PGE2 contents by enzyme immunoassay (EIA), and the cells were harvested to determine the cellular COX activity or to prepare the total RNA. For the detection of mRNA expression by RT-PCR and Northern blot analyses, the harvested cells were immediately frozen in liquid N2 and then stored at -80°C.

Assay for PGE2 measurements and determination of cellular COX activity

The PGE2 concentration in the conditioned media was assessed by an EIA kit (Cayman). The cellular COX activity was measured by the conversion of [14C]arachidonic acid to PGE2. In brief, the fibroblasts obtained as above were washed 3 times with ice-cold phosphate-buffered saline (pH 7.4, PBS) and then suspended in 1 ml of Hank's balanced salt solution (Nissui). The reaction was initiated by the addition of 20 µM [14C]arachidonic acid (88,000 dpm) to the cell suspension. Samples were incubated in a shaking water bath at 37°C for 5 min, after which the reaction was terminated by adding 0.5 ml of 1 M HCl. The reaction products were extracted by the addition of 0.5 ml of saturated NaCl solution followed by 3 ml of ethyl acetate and then separated by thin-layer chromatography (TLC). The PGE2 fraction was scraped from the TLC plates, and the radioactivity was estimated by scintillation counting. COX activity was calculated from the radioactivity as pmole PGE2 produced per 106 cells.
Effect on microsomal COX enzymes from bradykinin-stimulated fibroblasts

To assess the susceptibility of COX enzymes in the fibroblasts to T-614 or indomethacin, microsomal membranes, which were thought to contain COX enzymes, were prepared from bradykinin-stimulated cells by the method of Koehler et al. (18). Briefly, the fibroblasts (1 x 10^8 cells) were cultured in five 150-mm dishes and stimulated with bradykinin as mentioned above. The cells were harvested and suspended in 5 ml of 100 mM Tricine buffer (pH 8.5). After sonication, microsomes were pelleted by centrifugation at 100,000 x g for 1 hr. The COX activity of the microsomes and drug effect on the activity were determined in a similar manner to that of standard COX-1 microsomes.

Detection of COX mRNA by RT-PCR

For determination of mRNA levels, total RNA was isolated from the above cells by the guanidinium thiocyanate method of Chomczynski and Sacchi (19). RT-PCR analysis was carried out with a slight modification of the techniques described by Milam et al. (20). In brief, reverse transcription was carried out as follows: A 1-μg sample of total RNA was added to a solution containing 50 pmoles of 3’ primer (see Table 1). The final volume of this mixture was adjusted to 7.5 μl with diethylpyrocarbonate (DEPC)-treated water, and the mixture was overlaid with 100 μl of mineral oil. The amplification conditions were held at 94°C for 1 min, 55°C for 2 min and 72°C for 3 min for appropriate cycles. Aliquots of PCR products were electrophoresed in 6% polyacrylamide gels in Tris borate/EDTA buffer (pH 8.0) and then stained with ethidium bromide. Quantitation of the bands was achieved by using a densitometer (AE-6900; Atto, Tokyo). For Southern blot analysis, electrophoresed PCR products were capillary blotted to a nylon membrane filter and detected with the ECL® (Amersham) 3’-labeled oligonucleotide probe (5’TGTTTGCAATCTTGTGC3’) for COX-2.

Detection of COX mRNA by Northern blot analysis

To further confirm the COX mRNA levels, the total RNAs were electrophoresed in 1.2% agarose-formaldehyde gels and then transferred onto nylon membrane filters (hybond-N, Amersham), as previously described (21). The aforementioned 24- and 23-mer of the 3’ primers (see Table 1) were used as the probes for COX-1 and 3'-actin, respectively. These oligonucleotides were 5'-end labeled with γ-32P-ATP (3000 Ci/mmol, Amersham) using a T4 polynucleotide kinase (Toyobo Co., Osaka). A 1.2-kilobase cDNA of murine COX-2 (Cayman) was used as a probe for COX-2 and labeled with α-32P-dCTP (3000 Ci/mmol, Amersham) using a multiprime DNA labeling system (Amersham). The blots were prehybridized in 50 mM phosphate buffer (pH 6.3) containing 50% formamide, 5× SSC, 1× Denhardt’s solution, 0.1% sodium dodecyl sulfate (SDS) and 100 μg/ml salmon sperm DNA for 4 hr at 42°C and then hybridized with the radiolabeled probes for 18 hr at 42°C. The blots hybridized with oligonucleotide probe were washed in 2× SSC-0.1% SDS at room temperature for 20 min and in 1× SSC-0.1% SDS at 42°C for 1 hr. In the case of the blots with the cDNA probe, the second washing was performed in 0.2× SSC-0.1% SDS at 60°C for 1 hr. Then the blots were dried and exposed to X-ray film for 3 days.

Statistical analyses

IC50 was calculated by the least squares method. The data were analyzed by Student’s t-test and taking a P value of < 0.05 as significant.

Table 1. Sequences of 5'- and 3'-primer of mouse COX-1, COX-2 or 3'-actin gene

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>5'-Primer (5' → 3')</th>
<th>3'-Primer (5' → 3')</th>
<th>Extended PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-1</td>
<td>TGGTACGCTTCCTGCCCACAGCT</td>
<td>AAGGACTAACCAGGACCGCCTCTGT</td>
<td>304</td>
</tr>
<tr>
<td>COX-2</td>
<td>TTTCAAAAGAAGATCTGGAAAGAAGT</td>
<td>GATCATCCTCTACCTGAGTGTCCTTT</td>
<td>304</td>
</tr>
<tr>
<td>3'-Actin</td>
<td>ACAACGGCTCAGGCATGTGCAA</td>
<td>GCTCAGGGGGAGCAATGCTTTC</td>
<td>969</td>
</tr>
</tbody>
</table>
RESULTS

Effect on standard enzyme preparations of COX-1 and COX-2

In all assays using three enzyme preparations, labeled arachidonate was mainly converted to PGE₂ and a small amount of PGF₂α was detected when the radioactive products were extracted, separated by TLC and visualized by autoradiography (data not shown). Since the production of PGs by these COX preparations reached a plateau for 5 to 10 min after adding the substrate, the effect of T-614 and indomethacin was examined 4 min after initiation of the reaction. As shown in Fig. 1, T-614 inhibited the enzyme activity of purified COX-2 in a concentration-dependent manner (IC₅₀: 7.7 μg/ml), whereas T-614 was neither active against COX-1 activity of the microsomal form nor the isolated form at concentrations up to 300 μg/ml. On the other hand, indomethacin inhibited both COX-1 and COX-2 activity, the IC₅₀ being 0.024–0.032 μg/ml and 0.12 μg/ml, respectively. Thus, T-614 was found to be a selective inhibitor of purified COX-2, and indomethacin was found to be a much more potent inhibitor of COX-1 than of COX-2.

Effect on bradykinin-stimulated PGE₂ production from fibroblasts

Bradykinin at 1 μM induced PGE₂ synthesis in mouse fibroblasts and enhanced the PGE₂ release to a level of more than 5 times in only 1 hr of culture, as compared with the basal levels. As shown in Fig. 2, the bradykinin-stimulated PGE₂ release was inhibited by T-614. The inhibition was apparent at a low concentration of 0.1 μg/ml and was more pronounced at high concentrations of 1 to 10 μg/ml. Indomethacin also potently inhibited the PGE₂ release at 0.1 μg/ml or less. These results agree closely with the previously reported findings (11). The inhibitory effect of T-614 was greatly superior to that predicted from the aforementioned cell-free enzyme assays. In order to explain this discrepancy, we attempted firstly to examine which of the COX enzymes was expressed in bradykinin-stimulated fibroblasts.

COX-2 expression and activity in bradykinin-stimulated fibroblasts

To take advantage of the RT-PCR method in a semi-quantitative manner, the appropriate number of PCR cycles was estimated with regard to COX-1 and COX-2 cDNAs, which were synthesized by reverse transcription from the total RNA extracted from the fibroblasts stimulated with bradykinin for 1 hr. The amplification product for COX-1 was visualized from 18 cycles, and the product for COX-2 was from 21 cycles. Furthermore, the amounts of each product increased up to 27 cycles (data not shown). As a consequence, the change in these fibroblasts and enhanced the PGE₂ release to a level of more than 5 times in only 1 hr of culture, as compared with the basal levels. As shown in Fig. 2, the bradykinin-stimulated PGE₂ release was inhibited by T-614. The inhibition was apparent at a low concentration of 0.1 μg/ml and was more pronounced at high concentrations of 1 to 10 μg/ml. Indomethacin also potently inhibited the PGE₂ release at 0.1 μg/ml or less. These results agree closely with the previously reported findings (11). The inhibitory effect of T-614 was greatly superior to that predicted from the aforementioned cell-free enzyme assays. In order to explain this discrepancy, we attempted firstly to examine which of the COX enzymes was expressed in bradykinin-stimulated fibroblasts.

![Fig. 1. Effect of T-614 and indomethacin on COX activities of standard enzyme preparations in vitro. Sheep seminal vesicle microsome enriched in COX-1 activity (A), COX-1 isolated from ram seminal vesicles (B) or COX-2 isolated from sheep placenta (C) was preincubated with a drug for 2 min at 37°C. Labeled arachidonic acid was then added, and the mixture was incubated for 4 min. Enzyme activity was measured by the conversion of arachidonic acid to PGs. Each point with a vertical bar represents the mean ± S.E.M. of the percent inhibition obtained from 3 separate experiments. The number in parentheses is the IC₅₀ value (μg/ml).](image-url)
Fig. 2. Effect of T-614 and indomethacin on PGE₂ production by fibroblasts stimulated with bradykinin. The cells were incubated for 1 hr with or without 1 μM bradykinin in the presence of a drug as indicated. The PGE₂ level in the culture media was determined by an EIA. Each column with a vertical bar represents the mean ± S.E.M. of 3 to 5 independent determinations. *P < 0.05, compared with the value of bradykinin alone.

Fig. 3. RT-PCR analysis of COX mRNAs and cellular COX activity before and after stimulation with bradykinin in fibroblasts. A: Time-course of COX-1 and COX-2 mRNA levels before and after the stimulation. The cells were incubated with 1 μM bradykinin (BK) for the time indicated and RT-PCR analysis was performed as described in Materials and Methods. The photograph is representative of 3 similar ones. B: Southern blot analysis of the corresponding PCR products shown in A by using an oligonucleotide probe (5'TGTGTGCATTCTTTCG3'). C: Cellular COX activity before and after the stimulation. The cells were incubated with or without 1 μM bradykinin for 1 hr, and each cellular COX activity was determined. Each column with a vertical bar represents the mean ± S.E.M. of 4 to 5 independent determinations.
mRNA levels was examined by carrying out PCR of 21 cycles for both COX-1 and COX-2. Regarding β-actin as an internal standard, PCR of 21 cycles was carried out similarly. Figure 3A shows the time-course of COX-1 and COX-2 mRNA levels before and after adding bradykinin to the fibroblast culture. The products of 304 bp for COX-1, which indicate the levels of the expression of COX-1 mRNA, remained unaltered upon incubation with bradykinin in comparison with those of the products of 969 bp for β-actin. On the other hand, the products of 304 bp for COX-2 were strongly induced by bradykinin; i.e., those did not appear before bradykinin addition, but were visualized within 30 min and remained positive for a further 30 min after that. Furthermore, Southern blot analysis was performed, and the blots were hybridized with an oligonucleotide probe specific to COX-2. As a result, only in the case of carrying out RT-PCR with the COX-2 primer, the bands appeared in size of 304 bp (Fig. 3B). Therefore, these results substantiate the fact that COX-2 expression is induced in fibroblasts after 1 hr incubation in the presence of bradykinin.

To further elucidate the COX-2 induction in fibroblasts, the cellular COX activity was measured after a 1 hr incubation with or without 1 µM of bradykinin. As shown in Fig. 3C, the conversion rate of arachidonic acid to PGE2 in the cells stimulated with bradykinin was significantly greater than that in the unstimulated cells, and the ratio of activities was about 1.4.

Effect on microsomal COX enzymes from bradykinin-stimulated fibroblasts

Figure 4 shows the effects of T-614 and indomethacin on COX activity of the microsomal membranes, which was prepared from the cells stimulated with bradykinin. Arachidonic acid was converted only into PGE2 in this assay-system, and the conversion reached a plateau in 30 min after addition of the substrate (data not shown). Therefore, the effect of each drug was examined 10 min after initiation of the reaction. T-614 inhibited the COX activity in a concentration-dependent manner, the IC50 being 48 µg/ml. Indomethacin, which had an IC50 of 0.061 µg/ml, inhibited this activity more strongly than T-614. Each inhibitory potency, as shown by the IC50 values, was midway between those against standard preparations of COX-1 and COX-2.

Effect on COX-2 induction

Figure 5 shows the effect of T-614 and indomethacin on COX-2 mRNA levels in the fibroblasts at 30 min following bradykinin addition. T-614, at 10 and 30 µg/ml, exhibited a dose-dependent inhibitory effect on COX-2 mRNA levels, the inhibition percents being about 20% and 50%, respectively. On the other hand, indomethacin at 10 µg/ml had no effect on COX-2 mRNA levels. The mRNA levels of COX-1 and β-actin were not changed remarkably by either treatment with T-614 or indomethacin.

To corroborate the COX-2 induction by bradykinin in the fibroblasts and its inhibition by T-614, we next performed Northern blot analysis by using the cells 30 min

![Graph](image-url)
**Fig. 5.** Effect of T-614 and indomethacin (IDM) on COX-1 (A) and COX-2 (B) mRNA levels in bradykinin-stimulated fibroblasts. The cells were incubated for 30 min with or without 1 μM bradykinin in the presence of a drug as indicated. RT-PCR analysis was performed as described in Materials and Methods. The photograph is representative of 2 similar ones.

**Fig. 6.** Northern blot analysis of COX-1 and COX-2 mRNAs in bradykinin-stimulated fibroblasts. The cells were incubated for 30 min with or without 1 μM bradykinin in the presence of a drug as indicated. Northern blot analysis was performed as described in Materials and Methods. The photograph is representative of 2 similar ones. IDM: Indomethacin.
after bradykinin addition. In planning the experiment for COX-2, we employed a multiprime-labeled cDNA probe, which was more sensitive than the 5’-end labeled probe, since the expression of this gene in cells stimulated for such a short time was expected to be markedly lower than the expressions of constitutive COX-1 and β-actin. As shown in Fig. 6, unstimulated fibroblasts had no significant amounts of COX-2 mRNA. However, when stimulated with bradykinin, the mRNA level was apparently detected at 30 min and abated in the presence of T-614 (30 μg/ml). Either the ineffectiveness of indomethacin on this level or unchanged expressions of COX-1 and β-actin were also confirmed again.

**DISCUSSION**

An increasing number of reports demonstrate the importance of an inducible COX, COX-2, in PGs production, which suggests that the identification of selective COX-2 inhibitors will lead to advances in the therapy of inflammation (22, 23); a selective inhibitor of COX-2 would exert antiinflammatory action without undesirable side effects such as gastric and renal damage, which are due to ability to inhibit COX-1, as seen with conventional NSAIDs. T-614 has been reported to exhibit antiinflammatory and analgesic effects at a dose range of 0.3–10 mg/kg, p.o., but found to have virtually no gastrointestinal ulcerogenic action even when 1000 mg/kg was orally given to rats (24). As its mechanism, T-614 inhibited PGs production in inflamed tissue, but not in non-inflamed tissue (11). The underlying precise mechanisms for the selective inhibition of PGs production, however, have hitherto remained unclear. Therefore, we examined the effect of T-614 on COX-2 with respect to both its activity and induction.

Our study using standard COX preparations suggests that the inhibitory effect of T-614 on COX activity was completely specific to COX-2. This may partly explain the antiinflammatory and analgesic effects of T-614 without any gastrointestinal toxicity. However, the concentrations (3 to 30 μg/ml) of T-614 inhibiting the purified COX-2 exceeded the physiological levels when administered to animals and humans, and they were relatively high when compared with the amount required for effective inhibition of PGE2 release from the intact cells, mouse fibroblasts stimulated with bradykinin. Previous experiments examining the inhibitory effect of T-614 on PGE2 generation by the cells have shown that 0.1 to 1 μg/ml of T-614 yielded detectable suppression of this PG release, the IC50 being 0.47 μg/ml (11). Furthermore, T-614, administered at 1 mg/kg or more, has been found to reduce the PGE2 contents in inflammatory exudates of rats, as well as indomethacin (11). Thus, it would be difficult to explain the selective and potent inhibition of PGs production by T-614 by only its ability to inhibit COX-2 activity. Accordingly, we drew attention to the effect of this drug on induction step of COX-2.

Mouse fibroblasts have been reported to induce COX-2 mRNA expression when subjected to phorbol esters (4), interleukin-1 (25) and platelet-derived growth factor (26). Bradykinin potentiates the PGs production in fibroblasts by increasing the release of arachidonic acid in mediating the activation of the phospholipase A2 (27) or phospholipase C pathway (28), as has been previously suggested. However, COX-2 induction by this peptide has not as yet been demonstrated. To assess the COX-1 and COX-2 mRNA levels, the RT-PCR method was firstly used since it was more sensitive than the Northern blot procedure (29). As a result, COX-1 mRNA was constitutively expressed in fibroblasts whether the cells were stimulated with bradykinin or not. COX-2 mRNA was not detected before incubation with bradykinin, but was expressed within 30 min following its addition. From the result of Southern blot analysis, the RT-PCR products by the COX-2 sensitive primer were corroborated to be derived from COX-2 mRNA. The COX-2 gene expression within such a short time was also confirmed by Northern blot analysis even though the level was lower than that of COX-1. In addition, the cellular COX activity increased apparently after 1 hr incubation with bradykinin, although not so remarkably. The microsomal COX activity prepared from the cells stimulated with bradykinin was suppressed by T-614, but the inhibition appeared weak in comparison with that against the purified COX-2, which indicated that the microsomal activity was a mixture of COX-1 and COX-2. These data strongly suggest that COX-2 expression is induced also by bradykinin in the fibroblasts, and this induction occurs in only 1 hr after contact with the peptide.

T-614 reduced the COX-2 mRNA levels 30 min after bradykinin stimulation in a concentration-dependent manner. The inhibitory effect of T-614 on COX-2 induction may be independent of any intervening protein synthesis because the COX-2 gene is a primary response gene (30). This finding was of special interest, since T-614 was found to inhibit the interleukin-1β production by virtue of suppression of the mRNA expression in human monocytic cells (21). Up to the present, inhibition of COX-2 at the induction level has been reported for dexamethasone (31), cyclosporin A (32) and sodium salicylate (33). On the other hand, conventional COX inhibitors including indomethacin have been shown to have no apparent effect on COX-2 induction in general (33, 34). T-614 exhibits steroid-like action in several experimental animal models for rheumatoid arthritis (24, 35) and shows immune-suppressive effects such as the inhibition of lymphocyte
proliferation and antibody production (36). Thus, the characteristic action of T-614 on COX-2 induction, similar to those of steroids and immunosuppressants, might support its value as an antirheumatic drug rather than its use as an NSAID.

As regards to the pharmacological properties of T-614, we have reported that this novel drug possesses anti-inflammatory effects different from those of NSAIDs (24). In addition to its minimum toxicity in rats, T-614 was found to be less effective on the writhing responses in mice and on the ultraviolet-induced erythema in guinea pigs. These acute inflammatory and pain models have been put to good use for the screening of NSAID action, but PGs, which would be involved in the development of the symptoms, may consist of only the COX-1 products. Therefore, T-614, which selectively inhibits the COX-2 activity and reduces its induction, might have no effect on the animal models.

In summary, the present in vitro studies demonstrate that T-614 selectively inhibits COX-2 activity and exhibits a reducible effect on the induction of COX-2 mRNA by bradykinin in fibroblasts. Therefore, it is suggested that at least some of the inhibitory effects of T-614 on PGE₂ production are mediated by the synergy of the inhibition of COX-2 activity and the inhibition of induction, and the mechanisms involved apparently differ from those of conventional NSAIDs. Such action of T-614 may explain the discrepancy in pharmacological properties between this drug and NSAIDs.

REFERENCES

24 Tanaka K, Shimotori T, Makino S, Aikawa Y, Inaba T, Yoshida C and Takeo T: Pharmacological studies of the new...
antiinflammatory agent 3-formylamino-7-methylsulfonylamino-6-phenoxy-4H-1-benzopyran-4-one. 1st commun.: Antiinflammatory, analgesic and other related properties. Arzeneimittel-forschung 42, 935–944 (1992)


