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On the Induction of Cyclooxygenase-2, Inducible Nitric Oxide Synthase and Soluble Phospholipase A_2 in Rat Mesangial Cells by a Nonsteroidal Anti-Inflammatory Drug: The Role of Cyclic AMP

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ABSTRACT

One of the challenges in the therapy with anti-inflammatory drugs is the avoidance of gastrointestinal side effects, which may be achieved by selective inhibition of cyclooxygenase (COX) -2. CGP 28238 is reported with these characteristics inhibiting selectively the COX-2 activity at nanomolar concentrations. However, we report here on a novel action of this compound uncovered during the application of higher concentrations. In rat mesangial cells, CGP 28238 induced the mRNA and the protein of COX-2 as well as those of inducible nitric oxide synthase and soluble phospholipase A_2. In the case of COX-2, this stimulation had no effect on the production of COX-2 metabolites because of the effective blockade of the enzyme. In contrast, the level of NO produced by the cells increased in a concentration-dependent manner from 1.2 to 12.5 nmol of nitrite/3 $\times$ 10^5 cells. Furthermore, in combination with low doses of IL-1 CGP 28238 superinduced the formation of nitrite. The observed effects were independent of the inhibition of prostaglandin formation, as suggested by the failure of the potent COX inhibitor diclofenac to cause similar effects. Furthermore, the activity and expression of enzymes downstream of the COX step, such as prostacyclin synthase, were unaffected by CGP 28238. The inductive action of CGP 28238 could be blocked by inhibitors for tyrosine kinases and protein kinase A, such as genistein and KT5720, respectively. The increase in intracellular cAMP concentration in rat mesangial cells and the inhibition by CGP 28238 of phosphodiesterase 4 activity with an IC_{50} value of 23 μM gave a rationale to explain the underlying mechanisms for the induction of the inflammatory response genes COX-2, soluble phospholipase A_2 and inducible NO synthase in rat mesangial cells.

COX is the pharmacological target of aspirin and other NSAIDs, which are therefore commonly used in the treatment of inflammatory diseases. Although low doses of NSAIDs inhibit the biosynthesis of prostaglandins, high concentrations interfere with processes not dependent on prostaglandins. Inhibition of neutrophil functions (Abramson *et al.*, 1985), inhibition of oxidative phosphorylation in mitochondria (Whitehouse and Haslam, 1962), disruption of signal transduction and the consequent interference with mobilization of intracellular calcium and alteration of protein kinase C activity (Abramson and Weissman, 1989; Abramson, 1992) have all been reported. More recent studies indicate that NSAIDs may exhibit some proinflammatory features *in vitro*, such as the increased potency of the LPS-stimulated release of IL-1 activity (Bahl *et al.*, 1994) or the generation of superoxide production in human neutrophils (Twomey *et al.*, 1989). Furthermore, NSAIDs have been shown to inhibit the proliferation rate, to alter the cell cycle regulation, and to induce apoptosis in cancer cell lines independent from prostaglandin pathways (Hanif *et al.*, 1996; Shiff *et al.*, 1996). Because these effects are concentration-dependent, the variations in dose and pharmacological kinetics of different NSAIDs may explain some of the variability in the response to NSAIDs.

CGP 28238 [6-(2,4-difluorophenoxy)-5-methyl-sulfonyl-amino-1-indanone)] is an anti-inflammatory compound that belongs to the group of methansulfonilides. In a previous study, we discussed the selective COX-2 inhibitory profile of this drug (Klein *et al.*, 1994), exhibiting potent anti-inflammatory, analgesic, and antipyretic properties without the

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ABBREVIATIONS: COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; LPS, lipopolysaccharides; IL-1, interleukin-1β; PDE, phosphodiesterases; $sPLA_2$, soluble phospholipase A_2; iNOS, inducible nitric oxide synthase; IBMX, 3-isobutyl-1-methylxanthine; RMC, rat mesangial cells; PCS, prostacyclin synthase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; RT-PCR, reverse transcriptase-polymerase chain reaction.

ulcerogenic effects associated with the use of current NSAIDs (Wiesenberg-Böttcher *et al.*, 1989), all of which inhibit COX-1 and COX-2 more or less unselectively (Brideau *et al.*, 1996). Whereas the specific inhibition of COX-2 occurs at subnanomolar concentrations, we report here on the potency of CGP 28238 to induce inflammatory response genes, namely COX-2 AND $sPLA_2$, and iNOS at micromolar doses.

Several cell types and tissues express the constitutive isoforms of NOS and phospholipase A_2, as enzymes believed to be involved in the maintenance of normal cellular physiology, in a manner comparable with that of COX-1; the inducible form is expressed in a manner similar to that of COX-2 after stimulation with a variety of proinflammatory agents, including LPS, cytokines, and diverse mitogens (Pfeilschifter *et al.*, 1989; Moncada *et al.*, 1991). In this context, it is surprising that CGP 28238 is both an inhibitor of COX-2 activity and a stimulator of COX-2 expression. We therefore undertook a study to investigate the mode of action of this compound on COX-2, $sPLA_2$, and iNOS expression.

Materials and Methods

Materials. CGP 28238 and CGP 28237 were synthesized by Dr. P.G. Ferrini, Ciba, Basel, Switzerland. L-745337, a kind gift of Dr. A. Ford-Hutchinson, Quebec, Canada. Cell culture media were obtained from PAA (Coelbe, Germany). Recombinant human IL-1 was kindly provided by Dr. K. Vosbeck (Ciba, Basel, Switzerland). COX-2 polyclonal antibodies and the $sPLA_2$ assay kit were from Cayman (Ann Arbor, MI). The polyclonal iNOS antibody was a kind gift of Dr. Lapetina (Molecular Cardiovascular Research Guide, Cleveland, OH). Rat PCS polyclonal antibodies were raised against a sequence (LNPDGSEKKDFYKDGK) homolog to the rat enzyme and synthesized as a multiple antigene peptide-octamer (Tam, 1988). The electrochemiluminescence kit, nitrocellulose membranes (Hybond-C), and cAMP kits were purchased from Amersham (Braunschweig, Germany). The NSAIDs sulfanilamide, naphthylethylenediamine, forskolin and IBMX were purchased from Sigma (Deisenhofen, Germany). Primers were synthesized by MWG-Biotech, (Ebersberg, Germany), AmpliTaq polymerase was obtained from Perkin-Elmer (Weiterstadt, Germany) and Superscript reverse transcriptase from GIBCO (Eggenstein, Germany). Genistein and KT5720 were purchased from Calbiochem (Bad Soden, Germany).

Cell culture. RMC were cultured as described previously (Klein *et al.*, 1994) and used between passages 5 and 10. To promote and maintain growth the cultures were kept under the following conditions: RPMI 1640 (10% fetal calf serum) supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), sodium selenite (5 μg/ml), L-glutamine (1%), penicillin (100 units/ml), and streptomycin (100 μg/ml). Growth was arrested by low serum conditions (0.5% fetal calf serum) and the absence of supplements for 24 hr. The cells were stimulated under low serum conditions with various concentrations of NSAIDs or 1 nM IL-1 for 24 hr. The supernatants were collected for determination of accumulated nitrite by the Griess reagent as described previously (Klein *et al.*, 1994).

Apoptosis. DNA was extracted from RMC cultured on 150-mm diameter culture dishes stimulated with NSAIDs by the Nucleon kit (Scotlab, Wiesloch, Germany) and analyzed on a 1% agarose gel for appearance of DNA laddering.

Nuclei of cells grown on cover slips were stained with the DNA-specific fluorochrome bisbenzamide trihydrochloride (Hoechst 33258). After fixing the cells with ice-cold acetone/methanol (1:3) for 30 min, cells were stained with 170 μM bisbenzamide and observed by fluorescence microscopy.

Northern blot analysis. Confluent RMC were cultured in 150-mm diameter culture dishes. After stimulation, the cells were washed twice with PBS (137 mM NaCl, 27 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$,1.4 mM KH_2PO_4) and harvested with a rubber policeman. Total cellular RNA was extracted from the cell pellet using the guanidinium thiocyanate/cesium chloride method (Sambrook *et al.*, 1989). Samples of RNA (20 μg) were separated on a 0.8% agarose gel containing 6.6% formaldehyde before their transfer to gene screen membranes (New England Nuclear, Boston, MA). After baking at 80° for 2 hr and prehybridization for 4 hr at 42° in 50% (v/v) formamide, 10% (w/v) dextran sulfate, 1% SDS (w/v), 1 M NaCl and 100 μg/ml denatured herring sperm DNA, the filters were hybridized for 16–18 hr at 42° to a ^{32}P-labeled cDNA probe coding for mouse $sPLA_2$ that is composed of residues 529-2390 (Walker *et al.*, 1996). After washing, the signal was detected by autoradiography.

Determination of intracellular cAMP concentrations and PDE activity. RMC (3×10^6 cells) were preincubated for 30 min with 100 μM CGP 28238, L-745337, NS-398 or 5 μM rolipram as a control. Adenylate cyclase activity was initiated by the addition of 5 μM forskolin. After an incubation for 30 min at 37°, cells were washed with PBS and intracellular cAMP was extracted twice with 500 μl of ice-cold ethanol (65%) containing 500 μM of the nonspecific PDE inhibitor IBMX. The solvent was evaporated and cAMP determined by enzyme immunoassay analysis according to the nonacetylation protocol of the supplier (Amersham).

Separation of PDE isoenzymes were carried out as described previously (Schudt *et al.*, 1991). Briefly, rat cardiac ventricles tissue (PDE2) were homogenized and the supernatant of the 25,000 $\times$ *g* centrifugation step was purified by a column containing Q-sepharose fast flow. PDE1 purification from bovine brain was done as outlined by Schudt *et al.* (1991). Isolated isoenzyme fractions for PDE3 and PDE5 were chromatographically prepared from human thrombocytes and human polymorphonuclear leukocytes served as the source for PDE4.

Whole cell RT-PCR analysis and western blot analysis. RMC (1.5×10^6) were lysed by osmotic shock in 100 μl of diethyl pyrocarbonate treated water and the RNA-containing supernatant was used for RT-PCR analysis as described previously (Nüsing *et al.*, 1996). Cell pellets were solubilized in PBS containing 1% Triton X-100 and equal amounts of protein were separated by a 10% SDS-polyacrylamide gel electrophoresis. Immunoblot analysis for COX-2 and iNOS were performed as described previously (Klein *et al.*, 1994). For detection of prostacyclin synthase, an IgG- and affinity-purified polyclonal antibody was used 1:100 for Western blot analysis.

Results

RMC demonstrate abundant induction of inflammatory response genes like COX-2 (Klein *et al.*, 1994; Rzymkiewicz *et al.*, 1995) and iNOS (Pfeilschifter *et al.*, 1992) after IL-1 stimulation. Therefore, we used this cell type as an easy, amenable approach for screening diverse NSAIDs for potential COX-2 selectivity (Klein *et al.*, 1994). In experiments coincubating IL-1 with COX inhibitors, it was evident that in addition to its inhibitory effect on COX-2, CGP 28238 exhibited further action on the IL-1-induced nitrite production. Table 1 summarizes the influence of different concentrations of CGP 28238 on prostanoid and NO synthesis in RMC coincubated with 1 nM IL-1 for 24 hr. At concentrations as low as 1 μM, CGP 28238 was able to induce significant up-regulation of NO formation as determined by the accumulation of the stable metabolite nitrite. Notably, considering the amount of prostanoids synthesized under these conditions, maximal inhibition (96–100%) of cylooxygenase-2 was given. As a control, diclofenac exerted potent inhibition on COX-2 activity but had no effect on NO synthesis. Moreover, with higher concentrations of diclofenac, even a slight decrease in nitrite production was observed.

By virtue of the simultaneous up-regulation of COX-2 and

TABLE 1

Influence of CGP 28238 on NO production and COX-2

Coincubation of RMC with IL-1 (1 nM) and different concentrations of CGP 28238 or diclofenac for 24 hr. Nitrite was detected by the Griess reagent. In parallel, in the supernatant 6-keto-$PGF_{1\alpha}$ has been measured as a parameter of COX activity by gas chromatography/tandem mass spectrometry. The table depicts the means ± standard error of four experiments of the same cell passage. Student's *t* test was performed comparing IL-1 data with coincubated samples.

		nmol of NO_2^-/ 3×10^5 cells	*p*	COX activity
				%
Control		0.71 ± 0.02		
Interleukin 1 (1 nM)		6.58 ± 0.17		100
IL-1 + CGP 28238	10nM	6.15 ± 0.29	n.s.	84.4 ± 10.3
IL-1 + CGP 28238	100nM	6.67 ± 0.12	n.s.	14 ± 13
IL-1 + CGP 28238	1μM	7.32 ± 0.34	<0.05	14 ± 2.7
IL-1 + CGP 28238	10μM	17.5 ± 0.81	<0.001	0
IL-1 + Diclofenac	1μM	6.75 ± 0.42	n.s.	0
IL-1 + Diclofenac	10μM	5.65 ± 0.52	n.s.	0

n.s., not significant.

iNOS in RMC, nitrite formation could be used as a representative parameter for the activation of early response genes. Continuing experiments showed the intrinsic effect of CGP 28238 on iNOS activity. (Fig. 1) Compared with indomethacin and diclofenac, which were both without effect (data not shown), CGP 28238 revealed its maximal inducing potency on RMC in the range of 100 μM (EC_{50} calculated to 44 μM). It should be noted that we often observed a large variability in the magnitude of CGP 28238-stimulated nitrite production at various cell passages, although this compound always had a marked and significant stimulatory effect.

It has been reported that in some cell types, NSAIDs cause apoptotic or cytotoxic processes in parallel with an increase of COX-2 protein or mRNA (Lu *et al.*, 1995). Therefore, we investigated this possibility by measuring DNA laddering and fluorochrome staining as well as by the ability of mesangial cells to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (a marker of intact mitochondrial metabolic activity) (Mosmann, 1983). CGP 28238 had no effect on these parameters (data not shown), excluding an apoptotic component in the action of CGP 28238.

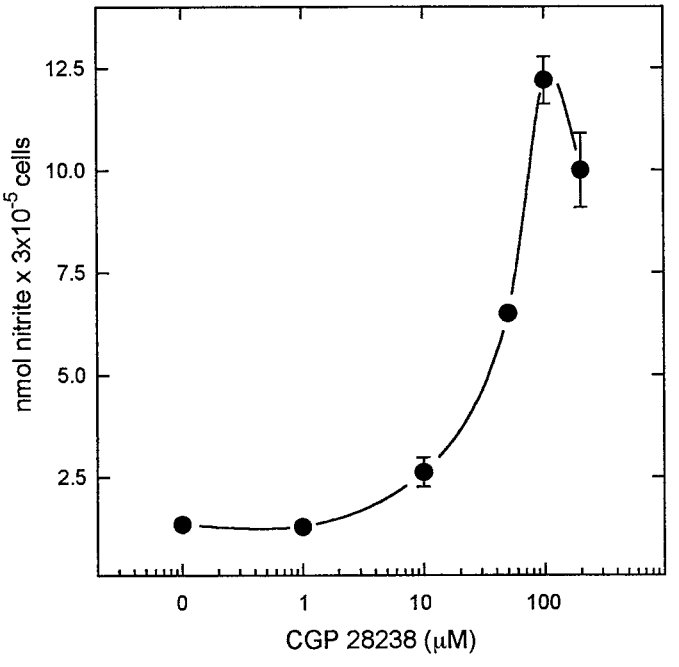


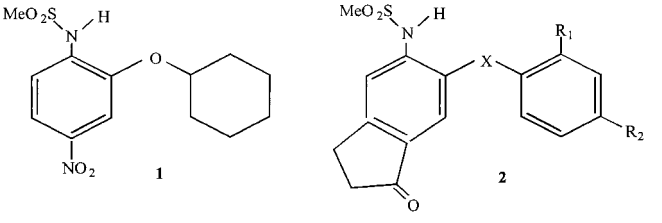
Fig. 1. Concentration dependency of CGP 28238 on NO production in RMC. Dose response of CGP 28238 on RMC. Cells were maintained in a fasting state for 48 hr and stimulated (24 hr) under low serum condition with the indicated concentrations of the compounds. The figure summarizes three independent experiments.

To determine whether the inductive effects were unique to this particular compound or shared by other related substances, we examined the effect of the selective COX-2 inhibitors NS-398 and L-745337 [a thioether-linked analogue of CGP 28238 (Vane and Botting, 1995) as well as CGP 28237, a nonhalogenated analogue (Böttcher *et al.*, 1987; Klein *et al.*, 1996). The data from Fig. 2 demonstrate that the ability to induce NO formation was restricted to CGP 28238, even though CGP 28237 also synergistically enhanced the IL-1 effect.

Next, we analyzed the molecular mechanism underlying the increase in NO formation by CGP 28238 for which protein and mRNA expression of iNOS were evaluated. Confluent RMC were treated with 100 μM CGP 28238 or IL-1 for 20 hr, and iNOS protein was detected by Western blot analysis. As shown in Fig. 3B, *lane 4,* iNOS protein was increased by CGP 28238, similar to the action exerted by IL-1 (Fig. 3B, *lane 2*). Corresponding results were obtained investigating COX-2 protein (Fig. 3A). Furthermore, the suppression of the induction by cycloheximide (Fig. 3, *lane 5*) indicated the necessity of *de novo* protein synthesis. The use of antibodies directed against prostacyclin synthase, an enzyme secondary to COX and assumed to be constitutively expressed in RMC (Klein *et al.*, 1995), revealed no differences in immunoblot analysis between CGP 28238 stimulated and control cells (Fig. 3C).

Performing RT-PCR analysis a massive stimulation of mRNA levels of both COX-2 and iNOS was observed (Fig. 4A) after CGP 28238 treatment. Hence mRNA expression was in line with the increased protein levels reported above. As a control, a fragment for both β-actin and prostacyclin synthase was amplified under identical conditions and remained unaltered by CGP 28238.

We reported recently (Mühl *et al.*, 1991) that IL-1 is able to stimulate the transcription of $sPLA_2$; consequently, the action of CGP 28238 on $sPLA_2$ expression was also studied. By means of Northern blot analysis (Fig. 4B), using a cDNA



Compound	Structure	X	R_1	R_2	Induct.	Superind
CGP 28238	2	O	F	F	+	+
CGP 28237	2	O	H	H	-	+
L-745337	2	S	F	F	-	-
NS-398	1				-	-
(Rolipram 5 μM)						+

Fig. 2. Structural comparison of different methylsulfonilides and their potency to induce NO synthesis in rat mesangial cells. The compounds were used in a concentration of 100 μM in combination with (superinduction of IL-1, 1 nM) or without IL-1. Nitrite was detected after 24 hr. (Rolipram as a specific PDE4 inhibitor served as a positive control for superinduction effects with IL-1.)

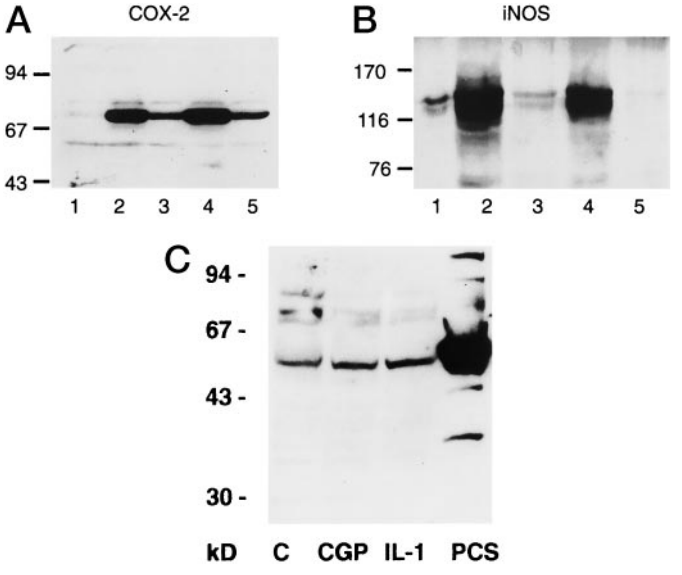


Fig. 3. Western blot analysis of CGP 28238 on COX-2, iNOS, and PCS. Immunoblot analysis of (A) COX-2 and (B) iNOS and (C) PCS in RMC. Cell lysates of CGP 28238 and IL-1 stimulated RMC were separated by SDS-PAGE and exposed to a polyclonal COX-2 antibody. *Lane 1*, control; *lane 2*, IL-1 (1 nM), *lane 3*, IL-1 in addition with 1 μM cycloheximide; *lane 4*, CGP 28238 (100 μM); *lane 5*, CGP 28238 + cycloheximide. C, Influence of control, CGP 28238, and IL-1 on prostacyclin synthase expression is demonstrated on RMC cell lysates. A partially purified probe of bovine PCS (Hara *et al.*, 1994) is given as positive control on the *right*.

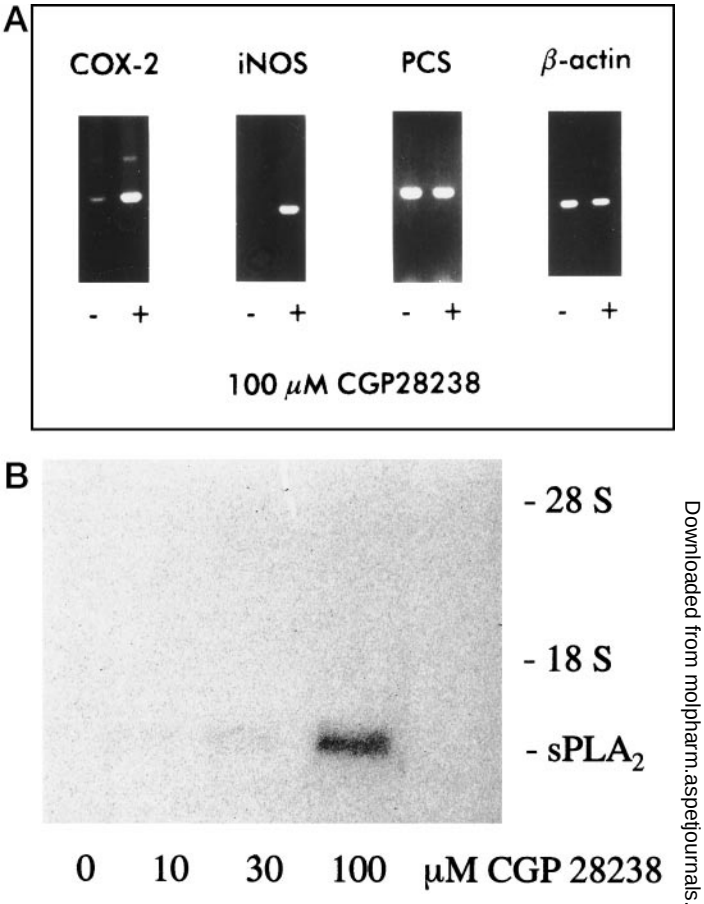


Fig. 4. mRNA analysis of COX-2, iNOS, PCS, and $sPLA_2$ from RMC after CGP 28238 stimulation. A, RT-PCR analysis of the mRNA of COX-2, iNOS, and prostacyclin synthase from RMC treated for 20 hr with 100 μM CGP 28238. B, Northern blot analysis of $sPLA_2$ mRNA in response to various concentrations of CGP 28238. Confluent RMC were exposed (20 hr) to the doses indicated and total mRNA (20 μg) was successfully hybridized to ^{32}P-labeled $sPLA_2$ probe as described in Methods. The densitometric analysis for hybridized mRNA revealed 0.38 arbitrary units for control, 0.33 for 10 μM, 2.67 for 30 μM, and 25.47 for 100 μM CGP 28238.

TABLE 2

$sPLA_2$ activity of RMC after CGP 28238 stimulation

After 24 hr incubation with the depicted concentration of CGP 28238 an aliquot of the supernatant of RMC (1.5×10^6 cells) was analyzed for $sPLA_2$ activity. A colometric based assay using a dithioanalog of phosphatidylcholine was applied. Data are presented as mean ± standard error.

		μmol/min/ml
CGP 28238	0μM	0.33 ± 0.01
CGP 28238	10μM	0.30 ± 0.02
CGP 28238	30μM	0.95 ± 0.09
CGP 28238	100μM	2.34 ± 0.07

probe for $sPLA_2$, a dose-dependent up-regulation of $sPLA_2$ message was observed, and the corresponding enzyme activity in the cell supernatant was increased as well (Table 2). This strengthened the concept of a general influence of CGP 28238 on mainly cytokine-responsive proteins.

In this respect, similarities between IL-1 and CGP 28238 concerning their molecular targets became obvious, and we addressed the question of whether similar or perhaps even identical signal transduction pathways were involved.

First, we used the tyrosine kinase inhibitor genistein to determine a possible participation of the tyrosine kinase pathway in the CGP 28238 mediated up-regulation of iNOS and COX-2. This compound was reported to suppress IL-1-induced NO and prostanoid production by inhibiting iNOS and COX-2 mRNA expression in RMC (Rzymkiewicz *et al.*, 1995). In accordance, genistein lowered IL-1-induced NO synthesis and a concentration of ~ 30 μM was necessary to obtain half maximal inhibition of NO release. CGP 28238-induced nitrite formation was also suppressed; however, we observed a >10-fold greater susceptibility to genistein (IC_{50} = 2.5 μM; Table 3).

From previous results on the existence of a cAMP-sensitive element involved in iNOS induction (Nüsing *et al.*, 1996), we evaluated whether the protein kinase A signaling cascade is involved in CGP 28238 induction of iNOS and COX-2 by making use of the inhibitor KT5720. Table 3 shows the respective IC_{50} values for KT5720 required to suppress nitrite accumulation in RMC stimulated with CGP 28238 or IL-1. The cAMP signaling pathway was reported by us as a functionally important mechanism in regulating the expression of COX-2 (Nüsing *et al.*, 1996), iNOS (Kunz *et al.*, 1994) and $sPLA_2$ (Pfeilschifter *et al.*, 1991) in RMC. Cyclic AMP has been shown to interact synergistically with IL-1 in triggering the expression of iNOS (Kunz *et al.*, 1994) and $sPLA_2$ (Mühl *et al.*, 1991). Because of this and the observed sensitivity of CGP 28238 to KT5720, we assessed the influence of the drug on cAMP accumulation after RMC stimulation. Using the diterpene forskolin to initiate adenylate cyclase activity, we found that the presence of CGP 28238 caused a 2-fold increase of intracellular cAMP levels. Rolipram, a selective inhibitor of the PDE type 4 isoenzyme, stimulated cAMP levels 4-fold, whereas the drugs L-745337 and NS-398 were without effect; similar effects were obtained without forskolin (Table 4). (The EC_{50} value of CGP 28238 in elevating the intracellular cAMP level in RMC was calculated to 3.8 μM.)

TABLE 3

Inhibition of NO synthesis in RMC by Genistein and KT 5720

IC_{50}-values for genistein and KT5720 on NO production in RMC were obtained by coincubating various concentrations of the inhibitors with the NOS inducers IL-1 (1 nM), CGP 28238 (100 μM), or the adenylate cyclase activator forskolin (5 μM) for 20 hr.

	Genistein	KT5720
Interleukin-1	29.4μM	>5 μM
CGP 28238	2.5μM	0.82 μM[a]
Forskolin	n.d.	1.25 μM[a]

[a] No 100% inhibition was obtained within the concentration range of 10 nM to 3 μM.

n.d., not done.

In a next step, the influence of different methylsulfonilides on their potency to inhibit multiple PDE isoforms was investigated. Table 5 depicts the representative IC_{50} values of CGP 28238, CGP 28237, and L-745337 on PDE isotypes 1–5 and shows preferable inhibition of the PDE subtypes 4 and 5 (IC_{50} = 6–31 μM), but only negligible influence on the isoforms 1, 2, and 3. We therefore considered the hypothesis that an inhibition of phosphodiesterases and the consequent up-regulation of intracellular cAMP could be responsible for the observed stimulatory effects of CGP 28238 in mesangial cells. It showed that the methylsulfonilides were nearly equipotent in suppression of PDE activity; however, they were totally different in their potency to up-regulate iNOS, COX-2, and $sPLA_2$. In this respect, interaction of CGP 28238 with further signal transduction pathways or an as-yet-unknown action of this drug was presumed. Sensitizing the cells by the use of rolipram with the consequent accumulation of intracellular cAMP, we observed a 2-fold stimulation of NO synthesis in combination with IL-1 or forskolin compared with the compounds used separately (Table 6). Interestingly, comparable action with a 2-fold extension of stimulation was demonstrated for CGP 28238 when used at lower concentrations (1–5 μM). In contrast, higher doses of CGP 28238 (50–100 μM) failed to significantly enhance NO synthesis in the presence of rolipram, probably because stimulation was maximal already.

Discussion

Cytokines, such as IL-1, tumor necrosis factor α, or bacterial LPS, have been shown to induce NO synthase, COX-2, and $sPLA_2$ in various cell types. Because of the fast response, these enzymes are considered to be coded by early response genes. The precise mechanisms by which these cytokines exert their effects on target cells are still largely unknown. However, diverse cytokine-specific pathways, such as ceramide signaling (Ballou, 1996) and mitogen-activated protein kinases (Ahlers *et al.*, 1994), have been postulated, leading to the activation of *cis*-acting elements in the respective promoter regions.

In this work, we report on the surprising capacity of a specific COX-2 inhibitor to induce COX-2, $sPLA_2$, and nitric oxide synthase protein and mRNA in RMC. Investigations with diverse standard NSAIDs, such as indomethacin and diclofenac, and more selective inhibitors, such as NS-398 and L-745337, failed to display similar properties. Thus, the action of CGP 28238 was not mediated by the suppression of tonically acting prostanoids (i.e., 6-keto-$PGF_{1\alpha}$ or PGE_2), as opposed to the recent reports for iNOS (Tetsuka *et al.*, 1994). Some controversy has surrounded the reporting of the modulating effects of NSAIDs on inflammatory stimuli, but some of these phenomena are definitely COX-independent because they occur only at concentrations above the minimum doses required to totally inhibit prostanoid generation. This was also obvious considering the high concentrations required to inhibit cell proliferation and induction of apoptosis (Shiff *et al.*, 1996).

Taking into account an IC_{50} value of ~20 nM for COX-2 inhibition, but because an EC_{50} of 44 μM is required for CGP 28238 elicited induction of COX-2, iNOS, and $sPLA_2$, a different action is obvious. It is worth pointing out that the enhancement of the IL-1- or rolipram-induced effects were already significant in the range of 1 μM; this proves that both the potentiation of IL-1 activity in low doses as well as the induction caused by CGP 28238 on its own were completely independent of the COX pathway.

A second major finding of this study was the observation that CGP 28238 inhibited PDE4 and -5 activity. Recently, it

TABLE 4

Effect of different compounds on intracellular cAMP levels

RMC were preincubated for 30 min with different methylsulfonanilides or rolipram after stimulation with forskolin (5 μM) or vehicle. Cyclic AMP levels (pmol/3 × 10^6) were measured 30 min after adenylate cyclase stimulation as described in Methods. The table describes the results of two different experiments. From two dose-response curves in the presence of forskolin, the EC_{50} of CGP 28238 in elevation of intracellular cAMP in RMC was calculated to 3.8 μM.

	in the presence of Forskolin	without Forskolin
Control	3.69 ± 0.17	0.15 ± 0.10
Rolipram (5 μM)	11.3 ± 0.65	0.92 ± 0.74
CGP 28238 (100 μM)	7.62 ± 0.95	0.59 ± 0.32
L-745337 (100 μM)	3.44 ± 0.81	0.20 ± 0.10
NS-398 (100 μM)	3.52 ± 0.42	0.23 ± 0.14

TABLE 5

Inhibition of phosphodiesterase isoenzymes by different methylsulfonanilides

PDE isoenzymes were chromatographically obtained from bovine brain, rat cardiac ventricles tissue (PDE2), human platelets (PDE3 and -5) or human polymorphonuclear cells (PDE4). Fractions containing preferentially one isoenzyme were used. IC_{50} values (μM) of the indicated compounds were determined in a reaction mixture containing 0.5 μM cyclic nucleotide/^{3}H-labeled cyclic nucleotide as outlined by Schudt *et al.* (1991).

	PDE1	PDE2	PDE3	PDE4	PDE5
CGP 28238	>100	>100	>100	23	14
CGP 28237	>100	79	>100	6	30
L-745337	n.d.	n.d.	>100	31	n.d.
Rolipram	>100	>100	>100	0.1	>100

n.d., not done.

TABLE 6

Effect of CGP 28238 on NO production during PDE inhibition by rolipram

Starved RMC were incubated with 5 μM rolipram for 30 min. NO synthesis was initiated with the compounds depicted in the table and nitrite was detected after a 24-hr incubation period. Values were expressed as the ratio of stimulated samples in the presence of rolipram compared with the appropriate controls (agents without rolipram). Rolipram alone increased nitrite synthesis by 133 ± 12% compared with control. The figure consists of the mean of four independent experiments ± standard error.

		% of control	*p*
CGP 28238	100μM	113 ± 5	n.s.
CGP 28238	50μM	104 ± 5	n.s.
CGP 28238	5μM	201 ± 14	<0.001
CGP 28238	1μM	241 ± 24	<0.001
Interleukin 1	1nM	191 ± 33	<0.050
Forskolin	5μM	211 ± 19	<0.001

n.s., not significant.

was demonstrated that PDE isoenzymes 1, 2, and 4 regulate cyclic nucleotide levels in intact mesangial cells (Ahn *et al.*, 1995) and PDE4 seems to be the predominant enzyme for hydrolysis of cyclic AMP. In contrast, the PDE5 subtype is responsible for the hydrolysis of cyclic GMP and was demonstrated to be absent in mesangial cells (Ahn *et al.*, 1995). In a previous work, we reported on the gradual induction of iNOS and COX-2 in RMC (Nüsing *et al.*, 1996) by membrane-permeable analogues of cAMP (dibutyryl-cAMP) and stressed the importance of cAMP as a central mediator in signal cascades of the early gene activation via cAMP-responsive elements in the respective promoter sequences. This interpretation is consistent with our notion of a cAMP involvement in the induction caused by CGP 28238. However, specific PDE inhibitors or nonspecific inhibitors, such as IBMX, had no effect on NO production when added alone, but markedly enhanced NO synthesis by IL-1 in a dose-dependent manner (Hirokawa *et al.*, 1994). This is in line with our experiments using IL-1 in combination with CGP 28238, CGP 28237, or rolipram (Fig. 2). Because of the rapid turnover of cAMP in intact cells, the intracellular accumulation of cAMP caused by PDE inhibitors can be considered as cellular priming events, requiring further stimuli triggering a complete response of target genes as evidenced by forskolin and IL-1. However, it could not be concluded that the action of CGP 28238 was mediated exclusively by the inhibition of PDE isoenzymes, because of the significant enhancement of NO formation by CGP 28238 in combination with rolipram (Table 6). This points to an additional intrinsic stimulating action of CGP 28238. Protein kinase A and cross-talk mechanisms involving tyrosine phosphorylation events are suggested as conceivable candidates (Table 3). One way to identify the molecular target of this underlying effect could be to investigate the promoter activities of the rat COX-2 or iNOS genes in RMC using a transient transfection method, as described recently for the human COX-2 gene (Inoue *et al.*, 1995).

One reason for the discrepancy to the structurally related drugs L-745337 and NS-398, which were equipotent in inhibiting PDE activity in polymorphonuclear leukocytes (L-745337), but without effect in RMC (neither induction of NO synthesis nor superinduction in the presence of IL-1 for NS-398 and L-745337), might be distribution or transport effects as well as more complex inhibitory mechanisms involved in the latter cell type. In a previous study, we emphasized the consequences of marginal modification of functional residues in these group of compounds, in this case resulting in a complete loss of COX-2 inhibition (Klein *et al.*, 1996).

Based on the current study, the clinical implications for application of COX-2 selective sulfonanilides are difficult to estimate. The action on PDE isoenzymes may be a useful intrinsic property of this class of compounds and contribute to the gastrointestinal safety of the drugs (Böttcher *et al.*, 1987; Vane and Botting, 1995), because plasma peak concentrations of 3 μM are easily reached after a single oral dose of 25 mg of CGP 28238 (Zimmerle *et al.*, 1991). Furthermore, some effects [e.g., the inhibition of superoxide production in activated human polymorphonuclear leukocytes by CGP 28238 (Zimmerle, *et al.* 1991)] can now be explained by the suppression of phosphodiesterases. On the other hand, considering the presence of cAMP-responsive elements in a large number of target genes (Yamamoto *et al.*, 1988), in addition to iNOS, COX-2, and $sPLA_2$, further action of this drug may be uncovered in additional studies and account for hitherto unexplained side effects. However, there is no evidence as yet from present studies (Wiesenberg-Böttcher *et al.*, 1989); moreover, it seems likely that the effects of CGP 28238 on mesangial cells were restricted to the rat species because of a lack of induction of NO synthesis in human mesangial cells or such different human cancer cell lines as DLD-1 or A549 cells (personal observation). In this context, however, it is necessary to note that induction of iNOS in human mesangial cells requires a mix of cytokines and no single cytokine that has been reported to induce iNOS in rat mesangial cells is able to trigger NO synthesis (Nicolson *et al.*, 1993). Furthermore, there are no reports as yet that describe cAMP as an inductor of iNOS in human systems.

The precise mechanism by which CGP 28238 regulates COX-2, iNOS, and $sPLA_2$ expression in RMC merits further investigation. This compound seems to be an interesting tool for distinguishing multiple pathways leading to the activation of diverse early response genes.

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