Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2384–2387, March 1991
Biochemistry

Aspirin inhibits interleukin 1-induced prostaglandin H synthase expression in cultured endothelial cells

(cyclooxygenase activity/nonsteroidal antiinflammatory drugs/cytokines/eicosanoids/inflammation)

KENNETH K. WU,* RADHIKA SANDUJA, AH-LIM TSAI, BURHAN FERHANOGLU, AND DAVID S. LOOSE-MITCHELL

Departments of Medicine and Pharmacology, Vascular Biology Research Center, University of Texas Medical School at Houston, Houston, TX 77030

Communicated by John Vane, December 26, 1990

ABSTRACT **Prostaglandin H (PGH) synthase (EC 1.14.99.1) is a key enzyme in the biosynthesis of prostaglandins, thromboxane, and prostacyclin. In cultured human umbilical vein endothelial cells, interleukin 1 (IL-1) is known to induce the synthesis of this enzyme, thereby raising the level of PGH synthase protein severalfold over the basal level. Pretreatment with aspirin at low concentrations (0.1–1 μg/ml) inhibited more than 60% of the enzyme mass and also the cyclooxygenase activity in IL-1-induced cells with only minimal effects on the basal level of the synthase enzyme in cells without IL-1. Sodium salicylate exhibited a similar inhibitory action whereas indomethacin had no apparent effect. Similarly low levels of aspirin inhibited the increased L-[^{35}S]methionine incorporation into PGH synthase that was induced by IL-1 and also suppressed expression of the 2.7-kilobase PGH synthase mRNA. These results suggest that in cultured endothelial cells a potent inhibition of eicosanoid biosynthetic capacity can be effected by aspirin or salicylate at the level of PGH synthase gene expression. The aspirin effect may well be due to degradation of salicylate.**

The diverse pharmacologic actions of acetylsalicylic acid (aspirin) are usually attributed to direct inhibition of catalysis by prostaglandin H (PGH) synthase (EC 1.14.99.1), a key enzyme in arachidonic acid metabolism (1–4). PGH synthase has two catalytic activities; a cyclooxygenase that catalyzes the oxygenation of arachidonic acid to form PGG_2 and a peroxidase that converts PGG_2 to PGH_2 (5). PGH_2 is the precursor molecule of several biologically active eicosanoids: PGE_2, PGI_2, thromboxane A_2, PGD_2, and $PGF_{2\alpha}$. It is generally accepted as a pharmacologic dogma that aspirin causes irreversible inhibition of the cyclooxygenase activity (and thus prostaglandin biosynthesis) by acetylating serine-506 of the PGH synthase molecule (6–8). However, earlier *in vivo* studies raised questions whether the pharmacologic activity of aspirin and other nonsteroidal antiinflammatory drugs is entirely mediated through this mechanism (1, 9).

PGH synthase has been found to have a rapid turnover rate (half-life of 5–6 min) in the few tissues examined (10, 11). Cytokines and phorbol diesters are known to induce synthesis of the enzyme, thereby raising the level of PGH synthase protein severalfold over the basal levels (11–14). In this communication, we report results of studies with cultured endothelial cells that indicate that aspirin and salicylate are able to inhibit the *de novo* synthesis of the enzyme by action at the level of gene expression.

MATERIALS AND METHODS

Materials. Recombinant interleukin 1β (IL-1) was obtained from Cistron (Pine Brook, NJ). Acetylsalicylic acid, sodium salicylate, acetaminophen, and indomethacin were obtained from Sigma. Iloprost was obtained from Amersham. PGE_2, $PGF_{2\alpha}$, and PGH_2 were purchased from Cayman Chemicals (Ann Arbor, MI). Purified ram seminal vesicle PGH synthase was obtained from Oxford Biomedical Research (Oxford, MI). Polyclonal antibody against the purified enzyme was prepared in rabbits and was affinity-purified. As described (11), this antibody recognized the 70-kDa subunit of PGH synthase in human umbilical vein endothelial cells (HUVECs). All the chemicals used were reagent grade.

Culture of Endothelial Cells. HUVECs were prepared from freshly obtained tissue as described (15, 16). The cells were cultured in flasks coated with porcine skin gelatin (1 mg/ml) and were maintained on medium 199 (GIBCO) containing 20% (vol/vol) heat-inactivated fetal calf serum, endothelial cell growth factor (50 μg/ml), heparin (100 units/ml), streptomycin (50 μg/ml), neomycin (200 units/ml), and penicillin (100 units/ml). We used HUVECs in passages 2–4 in all experiments.

Western Blot Analysis. The level of PGH synthase protein was analyzed by Western blot as described (11). In brief, monolayers of HUVECs in T-75 flasks (6×10^6 cells per flask) were lysed with phosphate-buffered saline containing 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.01% EDTA, and 0.03% leupeptin. The lysate was boiled for 3 min and centrifuged at 16,000 × *g* for 10 min in an Eppendorf microcentrifuge. The supernatant containing solubilized PGH synthase was analyzed by SDS/PAGE (17). The resolved proteins were transferred electrophoretically to nitrocellulose membranes and the blot was saturated with a solution of powdered milk. Visualization of the synthase band was achieved by successive exposure of the blot to the specific antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad), and finally chromogenic peroxidase substrates.

Assay of PGI_2. The quantity of PGI_2 in the HUVEC conditioned medium was measured as its stable metabolite, 6-keto-$PGF_{1\alpha}$ by a sensitive and specific radioimmunoassay as described (12).

Metabolic Labeling and Detection of L-[^{35}S]Methionine-Labeled PGH Synthase. The metabolic labeling procedure was as described (11). Confluent HUVECs in T-75 flasks were washed with methionine-free RPMI 1640 medium and then incubated with fresh medium containing L-[^{35}S]methionine (30 μCi/ml; 1 Ci = 37 GBq) at 37°C alone or with IL-1 (10 units/ml) for 2 hr. [^{35}S]Methionine-labeled PGH synthase was isolated by *Staphylococcus aureus* immunoprecipitation technique as described by Raz *et al.* (13), using polyclonal antibody against the synthase. The isolated enzyme was analyzed by SDS/PAGE, and the gels were dried and then exposed to Kodak XAR-5 x-ray film to visualize the labeled

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PG, prostaglandin; IL-1, interleukin 1; HUVEC, human umbilical vein endothelial cell.

*To whom reprint requests should be addressed.

synthase band. The radioactivity in the band was quantitated by densitometry of the x-ray films.

Northern Blot Analysis. Monolayers of confluent HUVECs in T-75 flasks were washed with ice-cold Hanks' buffer and lysed with 5 M guanidinium isothiocyanate. After sonication, the lysates were centrifuged at 25,000 × *g* for 24 hr through a 5.7 M CsCl cushion (18). The RNA pellets were resuspended in diethyl pyrocarbonate-treated water and extracted with an equal volume of phenol/chloroform/isoamyl alcohol, 25:24:1 (vol/vol), followed by precipitation with 0.1 vol of 3 M sodium acetate (pH 5.2) and 2.5 vol of absolute ethanol at −20°C for 16 hr. The RNA was pelleted by centrifugation at 16,000 × *g* for 10 min and then resuspended in diethyl pyrocarbonate-treated H_2O. The resulting RNA (20 μg) was denatured with 15 mM methylmercuric hydroxide, size-fractionated by formaldehyde/agarose gel electrophoresis, transferred to a nylon filter (GeneScreen, DuPont), and cross-linked by UV irradiation. The filter was prehybridized at 60°C for 3 hr in hybridization buffer containing 50% (vol/vol) deionized formamide, 1× Pipes buffer (pH 6.2), 0.5% SDS, and salmon sperm DNA (100 μg/ml) (1× Pipes buffer = 20 mM Pipes/2 mM EDTA/0.8 M NaCl). The ^{32}P-labeled PGH synthase cDNA probe (10^6 cpm/ml) was added to the blot and incubated at 55°C overnight. The blots were washed for two 20-min periods with 2× SSC/0.1% SDS at room temperature (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) and then with 0.1× SSC/0.1% SDS at 60°C for 20 min before the radioactive bands were visualized by exposure of the filters to Kodak XAR-5 film for 48 hr.

RESULTS AND DISCUSSION

We evaluated the effect of aspirin on the PGH synthase level maintained in the cultured cells by Western blot analysis. Our work (11) has shown that the HUVEC PGH synthase is present as a single 70-kDa band. In eight experiments, when aspirin was present at concentrations of 5 μg/ml or higher, we observed consistently a significant reduction in this 70-kDa band in quiescent and IL-1-treated cells (Fig. 1*A*) without any apparent effect on the endothelial-cell β-tubulin (Fig. 1*B*). At aspirin concentrations lower than 5 μg/ml, there was no significant change in synthase levels in the quiescent cells (Fig. 1*A*). On the other hand, marked inhibition of the induction of PGH synthase levels in IL-1-induced cells was observed at aspirin levels as low as 0.01 μg/ml (0.06 μM) and in some cases the inhibition appeared to be complete at 0.5 μg/ml (Fig. 1 *A* and *C*). Sodium salicylate also exerted a potent inhibitory effect on the level of synthase in IL-1-treated cells, giving partial inhibition at 0.01 μg/ml, whereas indomethacin at 50 μM had no apparent effect (Fig. 1*C*). Acetaminophen at 25 μg/ml also appeared to reduce the PGH synthase level. Incubation of the purified PGH synthase with salicylate did not decrease the intensity of the 70-kDa band on a subsequent Western blot, ruling out a direct artifactual alteration of the PGH synthase molecule by salicylate (data

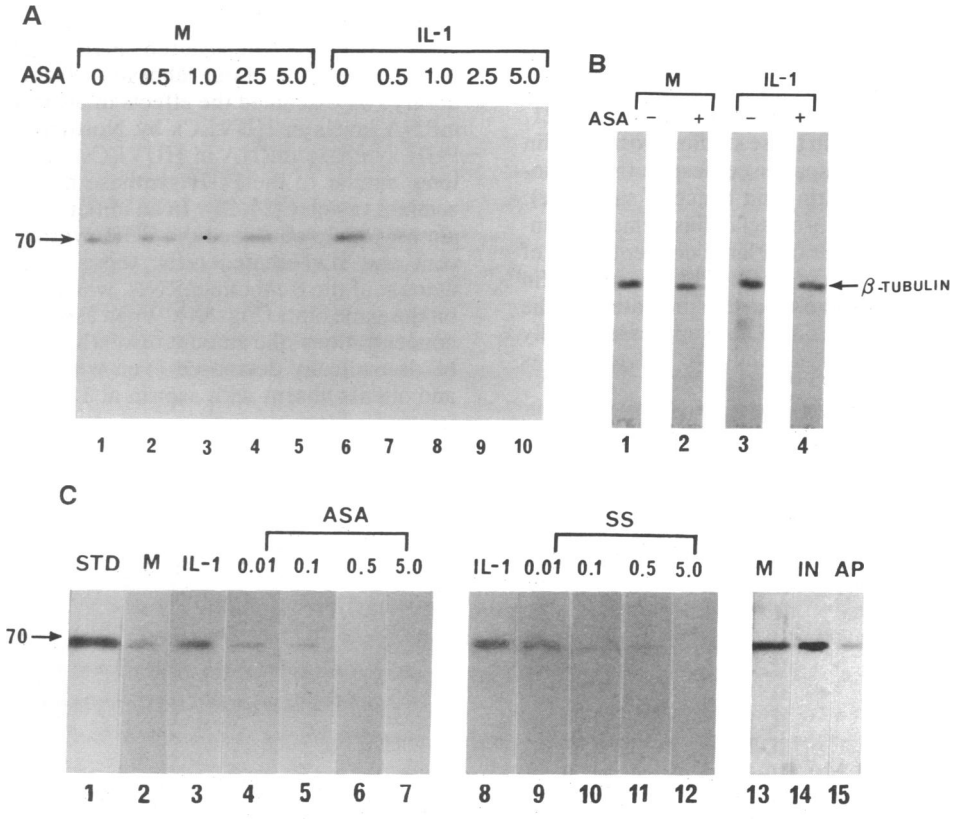

FIG. 1. (*A*) Western blot analysis of PGH synthase levels in cultured endothelial cells. Confluent HUVECs were preincubated with the indicated concentrations of aspirin (ASA, in μg/ml) at 37°C for 30 min. Cells were washed and then incubated with fresh medium alone (M) or IL-1 at 10 units/ml for 2 hr. Cells were lysed and PGH synthase levels were analyzed on Western blots. Representative data from eight experiments are shown. (*B*) Western blot analysis of HUVEC β-tubulin performed under the same conditions as in *A* except that a monoclonal antibody against β-tubulin was employed. Where indicated (+), aspirin was present at 25 μg/ml during the preincubation; otherwise it was absent (−). (*C*) Comparison of the effects of pretreatment with aspirin (ASA), sodium salicylate (SS), indomethacin (IN), and acetaminophen (AP) on PGH synthase levels. Lane 1 contained standard PGH synthase (STD, 60 ng); lane 2 was a sample from HUVECs incubated with medium alone (M) for 2 hr; lanes 3–7 were from HUVECs pretreated for 30 min with aspirin (0–5 μg/ml) followed by a 2-hr incubation with IL-1 (10 units/ml); lanes 8–12 were from HUVECs preincubated with the indicated concentrations of sodium salicylate before being stimulated with IL-1 for 2 hr; lane 13 was from HUVECs incubated in culture medium for 2 hr; lanes 14 and 15 were from cells pretreated with indomethacin (50 μM) and acetaminophen (25 μg/ml), respectively.

not shown). The addition of PGE_2 (10 μM), $PGF_{2\alpha}$ (10 μM), PGH_2 (5 μM), or iloprost (10 nM) to HUVECs was also found to have no effect on the amount of detectable synthase in the cells (data not shown). This makes it unlikely that the inhibitory action of aspirin or salicylate was due to blocking the formation of eicosanoids participating in a feedback stimulation of PGH synthase synthesis.

Burch *et al.* (19) estimated the IC_{50} of aspirin in acetylating endothelial-cell cyclooxygenase to be 18 μg/ml. At 1.8 μg/ml, they found aspirin to have no effect on the cyclooxygenase activity. To evaluate the effect of aspirin on the cyclooxygenase catalytic activity in our HUVECs, we analyzed prostacyclin synthesis in the cells as described in Table 1. Immunoreactive PGI_2 metabolite production in IL-1-treated HUVECs was inhibited 19% by aspirin at 0.18 μg/ml and 66% by aspirin at 1.8 μg/ml. Sodium salicylate exerted an effect similar to that of aspirin (Table 1). Thus, the cyclooxygenase activity in our IL-1-treated HUVECs was inhibited by aspirin and sodium salicylate levels of the same order as that found to decrease the amount of PGH synthase (see Fig. 1 *A* and *C*). The inhibitory action of sodium salicylate was of the same order of magnitude as aspirin. Since aspirin is unstable and rapidly converted to salicylate in aqueous solution, most if not all of the inhibitory effects of aspirin on PGH synthase levels may be attributed to salicylate. This hypothesis is in keeping with *in vivo* observations made in human and animal experiments. For example, Higgs *et al.* (20) showed a sustained suppression of PGE_2 concentrations in the inflammatory exudates of rats for several hours after oral aspirin administration, long after the aspirin had been totally converted to salicylate, and thus concluded that the antiinflammatory action of aspirin was due to salicylate. Similar observations have been made in human studies (21, 22). It is interesting to note that in these studies prostaglandin biosynthesis was not entirely suppressed despite the administration of a large dose of aspirin (and hence of salicylate). In one study (20), 30–40% of prostaglandins remained unsuppressed despite the presence of a large concentration of salicylate in the exudate (160 μg/ml). The prostaglandin biosynthesis in cultured endothelial cells stimulated by the inflammatory mediator IL-1 was also not suppressed totally by salicylate. In the presence of salicylate (18 μg/ml; as aspirin or sodium salicylate), IL-1-treated HUVECs were still capable of synthesizing 34% of the control levels of immunoreactive metabolite. Treatment of quiescent cells with salicylate (18 μg/ml) caused only a 5% reduction in the immunoreactive PGI_2 metabolite detected. The apparent resistance of a third of the PGI_2 synthesis in the IL-1-treated cells and almost all of the PGI_2 synthesis in the quiescent cells is surprising, and the reason for it is not clear. It may reflect an aspirin-resistant form of cyclooxygenase or the production of arachidonate metabolites by noncyclooxygenase processes of the sort recently described by Roberts and his colleagues (23).

Table 1. Effects of aspirin and salicylate on PGI_2 biosynthesis in quiescent and IL-1-stimulated HUVECs

Compound	Concentration, μg/ml	Stimulus	6-keto-$PGF_{1\alpha}$, ng/ml
Aspirin	0	Medium	1.77 ± 0.15
	18	Medium	1.68 ± 0.17
	0	IL-1	3.53 ± 0.24
	0.18	IL-1	2.87 ± 0.28
	1.8	IL-1	1.19 ± 0.26
	18	IL-1	1.19 ± 0.20
Salicylate	0	IL-1	3.45
	0.18	IL-1	1.94
	1.8	IL-1	1.91
	18	IL-1	1.20
Aspirin	0.18	IL-1	1.65
	1.8	IL-1	1.50
	18	IL-1	1.02

HUVECs were pretreated with aspirin or vehicle at 37°C for 30 min, washed, and then incubated with medium alone or with IL-1 (10 units/ml) at 37°C for 2 hr. Arachidonic acid (10 μM) was added and incubated for 10 min at 37°C. The conditioned medium was removed and the PGI_2 content was measured by RIA as its stable metabolite, 6-keto-$PGF_{1\alpha}$. Each value represents the mean ± SEM of three experiments. Additional experiments were performed under similar conditions to compare the effects of sodium salicylate and aspirin. Each value represents the mean of two experiments.

To determine whether the reduction of PGH synthase level elicited by aspirin was mediated through inhibition of *de novo* synthesis of PGH synthase, we investigated the effect of aspirin on L-[^{35}S]methionine incorporation into the synthase by using immunoprecipitation to isolate the protein. Aspirin was found to inhibit methionine incorporation in IL-1-treated HUVECs (Fig. 2). Densitometric analysis of the synthase radioactivity indicated that IL-1 increased the methionine incorporation by 2.2-fold compared to the medium control and that aspirin reduced the IL-1-induced methionine incorporation into PGH synthase in a concentration-dependent manner: by 5% at 0.01 μg/ml, 14% at 0.1 μg/ml, and 44% at 1 μg/ml. At 10 μg/ml, the incorporation was reduced by 56%, to almost exactly the level in the medium control. These results are in agreement with the enzyme activity experiment and further support the proposal that low concentrations of aspirin suppress the synthesis of PGH synthase in cytokine-induced cells.

By using a 2.1-kilobase (kb) PGH synthase cDNA probe recently obtained in our laboratory from a HUVEC cDNA library, we examined the effects of aspirin on PGH synthase mRNA levels in HUVECs by Northern blot analysis. The PGH synthase mRNA in HUVECs was found to be 2.7 kb long, similar to the PGH synthase mRNA reported in ram seminal vesicles (24–26). In an initial test, aspirin at 5 μg/ml almost totally abolished the PGH synthase mRNA in quiescent and IL-1-treated cells; there was no change in the amount of the β-tubulin mRNA, which was used as a control on the same blots (Fig. 3*A*). When aspirin was tested at lower concentrations, the amount of synthase mRNA was found to be dramatically decreased even with aspirin at 0.01 μg/ml and almost absent with aspirin at 1 μg/ml (Fig. 3*B*).

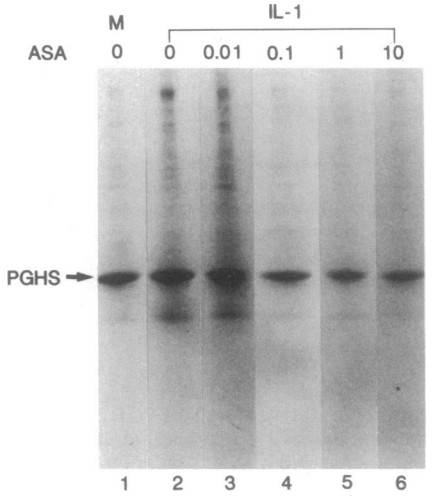

FIG. 2. Effect of aspirin on synthesis of PGH synthase (PGHS). Incorporation of [^{35}S]methionine into the 70-kDa subunit of the synthase was assayed. Cells were incubated in fresh medium alone (lane M) or with IL-1 (10 units/ml; lanes IL-1). The concentration of aspirin (ASA; in μg/ml) is shown at the top of each lane of the autoradiograph.

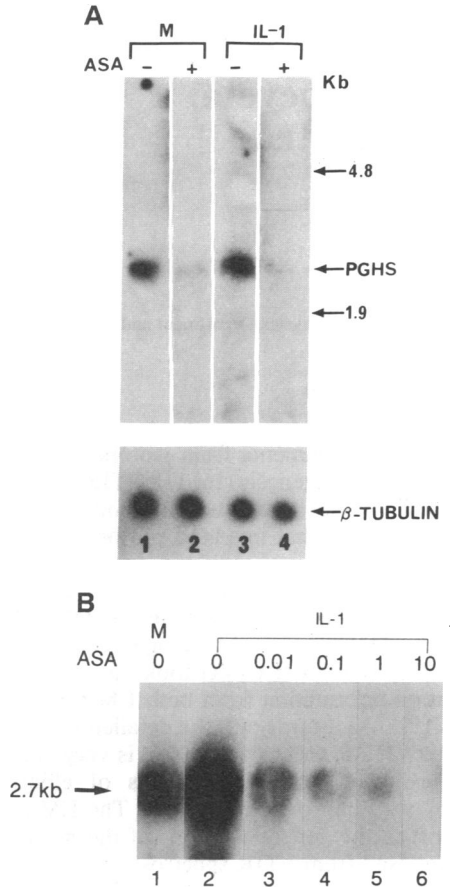

FIG. 3. Influence of aspirin on PGH synthase mRNA levels in quiescent and IL-1-treated HUVECs. (*A*) Autoradiographs of Northern blots hybridized with a 2.1-kb human PGH synthase cDNA probe. HUVECs were incubated for 2 hr with medium alone (M) or with IL-1 (IL-1) after pretreatment with (+) or without (−) aspirin (ASA; 5 μg/ml). rRNAs were used as molecular markers. The synthase cDNA probe hybridized to a single mRNA of 2.7 kb (indicated as PGHS). Autoradiograms from the same filters after washing and rehybridization with a β-tubulin cDNA probe are shown below. These autoradiographs are representative of data from four experiments. (*B*) Evaluation of the effects of aspirin concentration on synthase mRNA levels. Experimental procedures were as described above, with the indicated levels of aspirin (ASA; in μg/ml) used for pretreatment of cells in fresh medium (M) or with IL-1.

Thus these findings indicate that low levels of aspirin and salicylate inhibit the induction of PGH synthase expression by the cytokine IL-1 by actions at the mRNA level in HUVECs; higher levels of the agents may actually suppress some of the basal synthesis of the protein seen in the absence of the cytokine. Cytokines are key inflammatory mediators and their actions appear to be mediated by stimulating PGE_2 and PGI_2 biosynthesis in several cells including the endothelial cell (27–29). If it is a general phenomenon, the potent inhibitory effects of salicylate on synthesis of PGH synthase in cytokine-treated cells would allow control of the production of active eicosanoids at an inflammatory site without much affecting the basal levels of eicosanoids necessary for normal function in tissues such as kidney and stomach. Salicylate, hence, has the potential for antiinflammatory effects without renal or gastrointestinal toxicity when optimal doses are used.

Cytokine-induced vascular inflammatory changes are considered to be an important step in atherogenesis. Through its marked inhibition of inducible PGH synthase in endothelial cells, aspirin may act to limit the biosynthesis of inflammatory eicosanoids at the vascular wall without severely affecting the basal PGI_2 production needed to defend against platelet activation at the vascular wall. It remains to be discerned whether the observed inhibitory effect of aspirin on PGH synthase mRNA expression in endothelial cells is at the transcriptional or post-transcriptional level. Because aspirin inhibits cytokine-inducible PGH synthase, we suspect that its action may be at the level of transcription. With the availability of the PGH synthase genomic DNA (30), elucidation of the molecular mechanism by which aspirin and salicylate inhibit PGH synthase has become possible. Valuable information should be forthcoming on the action of aspirin, salicylate, and other nonsteroidal antiinflammatory drugs at the molecular level in endothelial cells and other tissues.

We thank Drs. Richard Kulmacz, Robert Lewis, and Nancy Lane for valuable discussions, Ms. Xiao-Ming Xu and Ms. Pam Polk for technical assistance, Dr. S. K. Sanduja for preparing the illustrations, and Ms. Nancy Fernandez and Ms. Gay S. Fullerton for secretarial assistance. The work was supported by grants from the National Institutes of Health (NS-23327 and HL-35387).

1. Flower, R. J. (1974) *Pharmacol. Rev.* **26,** 33–67.
2. Vane, J. R. (1971) *Nature (London) New Biol.* **231,** 232–234.
3. Smith, J. B. & Willis, A. L. (1971) *Nature (London) New Biol.* **231,** 235–237.
4. Needleman, P., Turk, J. & Jakschik, B. A. (1986) *Annu. Rev. Biochem.* **55,** 69–102.
5. Ohki, S., Ogino, N., Yamamoto, S. & Hayaishi, O. (1979) *J. Biol. Chem.* **254,** 829–836.
6. Roth, G. J., Sanford, N. & Majerus, P. W. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 3073–3076.
7. van der Ouderaa, F. J., Buytenhek, M., Nugteren, D. H. & van Dorp, D. A. (1980) *Eur. J. Biochem.* **109,** 1–8.
8. Roth, G. J., Machuga, E. T. & Ozols, J. (1983) *Biochemistry* **22,** 4072–4075.
9. Vane, J. & Botting, R. (1987) *FASEB J.* **1,** 89–96.
10. Fagan, J. M. & Goldberg, A. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2771–2775.
11. Wu, K. K., Hatzakis, H., Lo, S. S., Seong, D. C. & Sanduja, S. K. (1988) *J. Biol. Chem.* **263,** 19043–19047.
12. Frasier-Scott, K., Hatzakis, H., Seong, D. C. & Wu, K. K. (1988) *J. Clin. Invest.* **82,** 1877–1883.
13. Raz, A., Wyche, A., Siegel, N. & Needleman, P. (1988) *J. Biol. Chem.* **263,** 3022–3028.
14. Raz, A., Wyche, A. & Needleman, P. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1657–1661.
15. Jaffe, E. A., Nachman, R. L., Becker, C. G. & Minick, C. R. (1973) *J. Clin. Invest.* **52,** 2745–2756.
16. Gimbrone, M. A., Cotran, R. E. & Folkman, J. (1974) *J. Cell Biol.* **60,** 673–684.
17. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
18. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
19. Burch, J. W., Baenziger, N. L., Sanford, N. & Majerus, P. W. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5181–5184.
20. Higgs, G. A., Salmon, J. A., Henderson, B. & Vane, J. R. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1417–1420.
21. FitzGerald, G. A., Oates, J. A., Hawiger, J., Maas, R. L., Roberts, L. J., II, Lawson, J. A. & Brash, A. R. (1983) *J. Clin. Invest.* **71,** 676–688.
22. Heavey, D. J., Barrow, S. F., Hickling, N. E. & Ritter, J. M. (1985) *Nature (London)* **318,** 186–188.
23. Morrow, J. D., Harris, T. M. & Roberts, L. J. (1990) *Anal. Biochem.* **184,** 1–10.
24. DeWitt, D. L. & Smith, W. L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1412–1416.
25. Merlie, J. P., Fagan, D., Mudd, J. & Needleman, P. (1988) *J. Biol. Chem.* **263,** 3550–3553.
26. Yokoyama, C., Takai, T. & Tanabe, T. (1988) *FEBS Lett.* **21,** 347–351.
27. Dinarello, C. A. (1988) *FASEB J.* **2,** 108–115.
28. Rossi, V., Breviario, G., Ghezzi, P., Dejana, E. & Mantovani, A. (1985) *Science* **299,** 174–176.
29. Albrightson, C. R., Baenziger, N. L. & Needleman, P. (1985) *J. Immunol.* **135,** 1872–1877.
30. Yokoyama, C. & Tanabe, T. (1989) *Biochem. Biophys. Res. Commun.* **165,** 888–894.