Proc. Natl. Acad. Sci. USA
Vol. 74, No. 11, pp. 4955–4958, November 1977
Biochemistry

Prostaglandin regulation of macrophage collagenase production

(endotoxin/macrophage activation/prostaglandin synthesis/connective tissue)

LARRY M. WAHL, CHARLES E. OLSEN, ANN L. SANDBERG, AND STEPHAN E. MERGENHAGEN

Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014

Communicated by Earl P. Benditt, September 6, 1977

ABSTRACT **The production of collagenase (EC 3.4.24.3) by endotoxin-stimulated macrophages was significantly inhibited by indomethacin, indicating that prostaglandins (PGs) mediate this effect. Inhibition of collagenase production by indomethacin was overcome by addition of exogenous PGE_2 at 10 nM whereas the addition of 0.1 and 1.0 μM PGE_2 increased the enzyme production to 3 times that achieved by endotoxin. Although the addition of prostaglandin alone to macrophage cultures did not stimulate collagenase production, the simultaneous addition of PGE_1 or PGE_2 and endotoxin enhanced collagenase activity 2- to 10-fold. This increase was detectable at PGE concentrations of 10 nM and was optimal at 0.1–1.0 μM. $PGF_{2\alpha}$ had little effect on either the enhancement of collagenase production by endotoxin-stimulated macrophages or its restoration in cultures inhibited by indomethacin. Radioimmunoassay of prostaglandins in the culture media revealed that macrophages exposed to endotoxin secreted 40-fold more PGE_2 than did unstimulated cells. The increase in PGE_2 was detected 4 hr after exposure to endotoxin and was maximal at 14 hr. The peak PGE_2 concentrations in the culture media were similar to those of exogenous PGE_2 (about 10 nM) needed to restore collagenase production in indomethacin-treated cultures. These findings demonstrate the involvement of PGE in the endotoxin-activation of macrophages with resultant production of collagenase.**

Certain chronic inflammatory lesions are often accompanied by destruction of connective tissue. Increased levels of collagenase (1–6) and prostaglandins (7–9) have been identified in many of these lesions. The coexistence of these two components in sites of inflammation suggests that prostaglandins may influence collagenase production. Since we have previously shown that the macrophage, a prominent cell type in chronic inflammatory lesions, can be stimulated by endotoxin (10) or lymphokines (11) to produce collagenase, it was of interest to determine whether prostaglandins were involved in the activation of these cells. Here we report that the elevated levels of prostaglandin E_2 (PGE_2) produced by endotoxin-stimulated macrophages have a regulatory role in the production of collagenase by these cells.

MATERIALS AND METHODS

Culture Methods. Macrophages were obtained from mineral oil-induced peritoneal exudates in guinea pig and cultured as described (10). Activation of the macrophages was achieved by the addition of endotoxin (30 μg/ml) from *Escherichia coli* (055:B5 Difco Laboratories, Detroit, MI) to the cultures. Media were harvested daily from the cultures and frozen at −20° until assayed. Prostaglandins E_1, E_2, and $F_{2\alpha}$ (PGE_1, PGE_2, and $PGF_{2\alpha}$) (generously supplied by John Pike, Upjohn Co., Kalamazoo, MI) were dissolved in ethanol and added to the culture media. The highest final concentration of ethanol in the cultures never exceeded 0.1%. Indomethacin (Merck Sharp and Dohme Research Laboratories, Rahway, NJ) was dissolved in ethanol and added to culture media in a final concentration of 0.1% ethanol. Cultures contained indomethacin at final concentrations of 10, 1.0, and 0.1 μM.

Collagenase Assay. Collagenase (EC 3.4.24.3) activity in the culture media was assayed on [^{14}C]glycine-labeled collagen fibrils as described (10). The data presented are typical of at least three repeated experiments.

Prostaglandin Assay. Media to be assayed for prostaglandins were extracted and separated by the method of Auletta *et al.* (12). One volume of 1 M sodium citrate (pH 3.5) was added to 2 volumes of medium, followed by the addition of 12 volumes of chloroform. After vortex mixing for 15 sec, the aqueous phase was removed by aspiration and the organic phase was dried with a stream of air. Silicic acid columns for separation of the prostaglandins were prepared by adding a slurry of 0.5 g of silicic acid (100 mesh, Mallinckrodt Scientific Products, Columbia, MD) in 2 ml of solvent I (benzene/ethyl acetate, 60:40, vol/vol) to glass columns (Kontes, no. 7 size 22, Vineland, NJ). Ten milliliters of solvent I was added to the columns and allowed to drain through. The dried sample extracts were suspended in 0.2 ml of solvent III (benzene/ethyl acetate/methanol, 60:40:10, vol/vol) and then 0.6 ml of solvent I was added. The 0.8-ml samples and 0.2 ml of solvent I used to rinse the sample tubes were applied to the silicic acid columns. The columns were allowed to run dry, and 1 ml of benzene was added to remove extraneous lipids. PGA and PGB were eluted by addition of 6 ml of solvent I. PGE was eluted next with 14 ml of solvent II (benzene/ethyl acetate/methanol, 60:40:2, vol/vol), followed by elution of PGF with 4 ml of solvent IV (benzene/ethyl acetate/methanol, 60:40:20, vol/vol).

The PGE and PGF fractions were dried under a stream of air. $PGF_{2\alpha}$ concentrations were determined with an anti-$PGF_{2\alpha}$ serum (Clinical Assays, Inc., Cambridge, MA) that exhibited a 50% crossreactivity with $PGF_{1\alpha}$; the values obtained are expressed as $PGF_{2\alpha}$ equivalents. This antiserum was also used to quantitate PGE after conversion of PGE to PGF by reduction with sodium borohydride at pH 9.0 according to the method of Levine *et al.* (13). Fifty percent of PGE_2 is converted to the $PGF_{2\alpha}$ isomer. These results are expressed as PGE_2 equivalents. The prostaglandin recovery rates were determined by adding [^{3}H]PGE_2 and [^{3}H]$PGF_{2\alpha}$ (New England Nuclear, Boston, MA) to some of the samples. Recovery was 40–45% for $PGF_{2\alpha}$ and 35–40% for PGE_2. The values reported for PGE_2 and $PGF_{2\alpha}$ equivalents have been corrected for recovery. Duplicate samples were analyzed and similar results were obtained in several experiments.

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

Abbreviations: PGA, PGB, PGE_1, PGE_2, $PGF_{1\alpha}$, and $PGF_{2\alpha}$, prostaglandins A, B, E_1, E_2, $F_{1\alpha}$, and $F_{2\alpha}$.

Table 1. Inhibition of macrophage collagenase production by indomethacin

Addition at 0 hr	Addition at 24 hr	Collagenase activity, cpm
Control		2
Endotoxin		3313
Endotoxin + indomethacin		84
Endotoxin	Indomethacin	3214
Endotoxin + indomethacin	Indomethacin	78

Culture flasks (75 cm^2) contained 3×10^7 adherent macrophages in 10 ml of Dulbecco's modified Eagle's medium. Endotoxin (30 μg/ml) was added to some of the flasks on the first day of culture. Indomethacin (10 μM) was added to some of the cultures either with the endotoxin or 24 hr later. The media were changed daily, and the 48-hr media were dialyzed and lyophilized. The concentrated media were added to labeled reconstituted collagen fibers (3731 cpm/300 μg of substrate) and incubated for 16 hr. Collagenase activity was determined by the amount of label solubilized. The results represent the mean value of duplicate samples.

RESULTS

The production of collagenase by endotoxin-stimulated macrophages was significantly inhibited by indomethacin (Table 1). Although 10 μM indomethacin was used in most of our experiments, inhibition could be demonstrated with concentrations as low as 0.1 μM indomethacin. When indomethacin was added 24 hr after the endotoxin, it had no effect on the production of collagenase during the next 24 hr. Because the inhibition of collagenase production by indomethacin indicated the involvement of prostaglandins in this process, prostaglandins were added to macrophage cultures and their effect on collagenase production was determined. In the absence of endotoxin, prostaglandins were ineffective. However, in the presence of endotoxin, both PGE_1 and PGE_2 were found to enhance collagenase production 2-fold (Fig. 1); $PGF_{2\alpha}$ had little effect (data not shown). At a given concentration, PGE_1 and PGE_2 were equally effective in enhancing collagenase production. Increased collagenase activity could be demonstrated by the addition of 10 nM PGE_1 or PGE_2; maximal enhancement, which varied 2- to 10-fold among experiments, occurred at 0.1–1 μM. The collagenase produced 48 hr after the addition of endotoxin to the cultures could be increased if prostaglandins were added at 0 hr but not 24 hr after the addition of endotoxin (Table 2). Because the collagenase activity was increased 10-fold in this experiment when 1 μM PGE_1 was added with endotoxin, the time of incubation of the collagenase assay was shortened to 2 hr to prevent complete digestion of the substrate. Because of the short incubation period, the collagenase activity from cultures stimulated with endotoxin alone appears low.

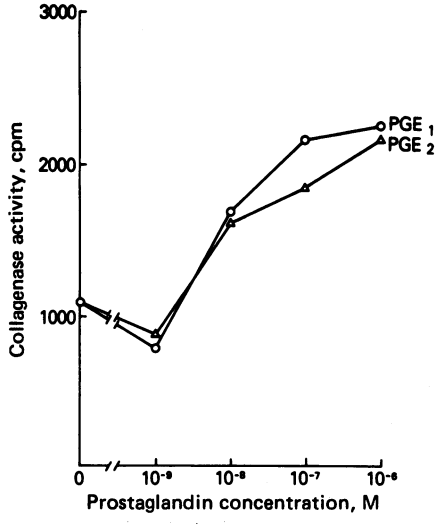

FIG. 1. PGE_1 and PGE_2 enhancement of production of collagenase by endotoxin-stimulated macrophages. The cells were plated and cultured as described in Table 1. Endotoxin (30 μg/ml) was added to the culture alone or in the presence of varying concentrations of PGE_1 or PGE_2. The concentrated media products were incubated for 4 hr in the presence of labeled collagen fibers (2763 cpm/225 μg of substrate) to determine collagenase activity.

Table 2. Effect of PGE_1 on endotoxin-induced macrophage collagenase activity

PGE_1 added at 0 hr	PGE_1 added at 24 hr	Collagenase activity, cpm
Endotoxin		167
Endotoxin + PGE_1		2233
Endotoxin	PGE_1	207
Endotoxin + PGE_1	PGE_1	2193

The cells were plated and cultured as described in Table 1. Endotoxin (30 μg/ml) was added to all cultures. Cultures received PGE_1 (1 μM) at 0 or 24 hr or both. The concentrated media products were incubated for 2 hr in the presence of collagen fibers (2586 cpm/225 μg of substrate) to determine collagenase activity.

Further evidence for the involvement of prostaglandins in the production of collagenase by endotoxin-treated macrophages was demonstrated by the restoration of collagenase activity in indomethacin-inhibited cultures by the addition of exogenous PGE_2 (Fig. 2). Similar collagenase activity was detected in the media of cultures that received only endotoxin and those that received endotoxin, indomethacin, and exogenous PGE (10 nM). The addition of PGE_2 at higher concentrations

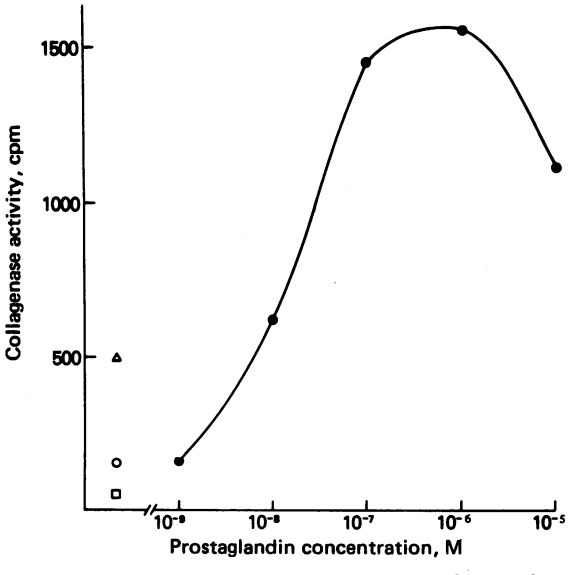

FIG. 2. PGE_2 restoration of collagenase production by endotoxin-stimulated macrophage cultures inhibited by indomethacin. The cells were plated and cultured as described in Table 1. The continuous line (●) indicates cultures that receive endotoxin, indomethacin (10 μM), and PGE_2 at the indicated concentrations. The three single points to the left of the curve indicate the collagenase activity in the media of cultures that received endotoxin (△), endotoxin and indomethacin (○), or nothing (□). The concentrated media products were incubated for 4 hr in the presence of labeled collagen fibers (1847 cpm/150 μg of substrate) to determine collagenase activity.

Table 3. Restoration of collagenase production in indomethacin-inhibited macrophage cultures by prostaglandins

Macrophage treatment	Collagenase activity, cpm
Control	12
Endotoxin	479
Endotoxin + indomethacin	114
Endotoxin + indomethacin + PGE_1	1084
Endotoxin + indomethacin + PGE_2	900
Endotoxin + indomethacin + $PGF_{2\alpha}$	267

The cells were plated and cultured as described in Table 1. Endotoxin (30 μg/ml) was added at 0 hr. Indomethacin (10 μM) was added either in the absence or presence of the indicated prostaglandins (1 μM). The concentrated media products were incubated for 4 hr in the presence of labeled collagen fibers (1504 cpm/150 μg of substrate) to determine collagenase activity.

(0.1–1.0 μM) resulted in a 3-fold increase in enzyme activity. However, the addition of 10 μM PGE_2 to the cultures caused a decrease in peak enzyme production. PGE_1 and $PGF_{2\alpha}$ also were examined for their ability to restore collagenase production that had been inhibited by indomethacin. At 1 μM, PGE_1 was as effective as PGE_2 in overcoming indomethacin inhibition of macrophage collagenase production, whereas $PGF_{2\alpha}$ had little effect (Table 3).

The culture media were assayed for prostaglandins by radioimmunoassay to determine if endotoxin-activation of macrophages was associated with an increase in prostaglandin production. A representative experiment demonstrating the PGE_2 production by macrophages in response to endotoxin is shown in Table 4. An increase in the concentration of PGE_2 in the culture medium was detected 4 hr after the addition of endotoxin. Maximal PGE_2 concentrations were reached by 14 hr. This response was completely inhibited by 10 μM indomethacin as well as lower concentrations (0.1 and 1.0 μM). These indomethacin concentrations also inhibit collagenase production. Although $PGF_{2\alpha}$ also increased in response to endotoxin, the concentrations were only 10–20% of those of PGE_2 (data not shown). The concentration of PGE_2 in the medium 4–14 hr after the macrophages were exposed to endotoxin increased from 0.6 ng/ml to 6.1 ng/ml.

DISCUSSION

Our data indicate that prostaglandins play an essential role in the production of collagenase by endotoxin-stimulated macrophages. This conclusion is supported by the following observations: (*i*) collagenase production by macrophages in response to endotoxin is inhibited by indomethacin, an inhibitor of prostaglandin synthesis; (*ii*) collagenase production can be restored in indomethacin-inhibited cultures by the addition of exogenous PGE_1 or PGE_2; (*iii*) collagenase production in response to endotoxin can be significantly enhanced by the addition of PGE_1 or PGE_2; (*iv*) radioimmunoassay of PGE_2 released into the culture media revealed that PGE_2 levels increased 40-fold in response to endotoxin; and (*v*) the maximal PGE_2 concentrations in the media of macrophages exposed to endotoxin are equal to or greater than those of exogenous PGE_2 (about 10 nM or 3.5 ng/ml) required to restore collagenase production in indomethacin-inhibited cultures to that achieved by endotoxin alone.

These findings demonstrate that PGE is involved in the initial events of macrophage activation that lead to collagenase production, because indomethacin or prostaglandins were effective when added with endotoxin but not 24 hr later.

Table 4. Effect of endotoxin on prostaglandin production by macrophages

Time, hr	PGE_2, ng/culture: Control	Endotoxin	Endotoxin + indomethacin
1	0.62	0.80	0.42
2	1.59	1.74	0.41
4	1.58	5.68	0.31
8	2.07	26.80	0.450
14	1.48	60.92	0.50
24	1.46	55.85	0.34
48	1.76	52.00	0.52

The cells were plated and cultured as described in Table 1. Some cultures served as controls while others received endotoxin (30 μg/ml) alone or in the presence of indomethacin (10 μM). Media were harvested at the indicated times and assayed for PGE_2 by radioimmunoassay. The results are presented as ng of PGE_2 equivalents/10 ml of culture medium and represent the mean from two cultures.

The prostaglandins required for activation were synthesized by the macrophages, as demonstrated by radioimmunoassay of the culture media. Production of prostaglandins by macrophages has been previously shown by Bray *et al.* (14). Our experiments revealed that macrophages exposed to endotoxin increased their production of PGE_2 by 40-fold.

The mechanism by which prostaglandins influence macrophages to produce collagenase may involve cyclic nucleotides. It is known that PGE increases cyclic AMP levels in various cell types. Previous studies have examined the involvement of cyclic AMP in macrophage function. Koopman *et al.* (15) demonstrated that PGE_1, PGE_2, β-adrenergic stimulators, aminophylline, or dibutyryl cyclic AMP added to macrophages with macrophage migration inhibition factor eliminates the action of this lymphokine. Additionally, macrophages stimulated by lymphocyte mediators have increased levels of adenylate cyclase, which catalyzes the formation of cyclic AMP from ATP (16), and macrophages exposed to PGE_1 have been reported to release cyclic AMP into the medium (17).

Several studies have demonstrated that elevated levels of collagenase and prostaglandins are found in chronic inflammatory lesions (1–9). High concentrations of prostaglandins and collagenase are secreted into the medium by isolated adherent rheumatoid synovial cells (18). Indomethacin does not inhibit ongoing collagenase production by either the macrophages stimulated *in vitro* (Table 1) or by the synovial cells that are presumably activated *in vivo* (18). The fact that this agent actually stimulates the production of the synovial cell enzyme may merely reflect differences in cell populations or culture conditions utilized.

In addition to the demonstration that prostaglandins regulate macrophage collagenase production, these findings also have important implications in immune reactions. Lymphocytes exposed to mitogens or specific antigens secrete products that stimulate macrophages to produce collagenase (11). Thus, it is likely that this mode of macrophage stimulation will also contribute to the increased concentrations of prostaglandins associated with inflammatory lesions. Prostaglandins have been reported to be inhibitory to *in vitro* lymphocyte activation (19, 20). Therefore, the prostaglandins produced by activated macrophages may serve as a regulator of further lymphocyte function, as suggested by their inhibition of the production of macrophage migration inhibitory factor (21).

1. Fullmer, H. M. & Gibson, W. A. (1966) *Nature*, **209**, 728–729.

2. Beutner, E. H., Triftshauser, C. & Hazen, S. P. (1966) *Proc. Soc. Exp. Biol. Med.* **121,** 1082–1085.
3. Harris, E. D., Jr. & Krane, S. M. (1974) *N. Engl. J. Med.* **291,** 557–563.
4. Harris, E. D., Jr. & Krane, S. M. (1974) *N. Engl. J. Med.* **291,** 605–609.
5. Harris, E. D., Jr. & Krane, S. M. (1974) *N. Engl. J. Med.* **291,** 652–661.
6. Krane, S. M. (1975) *Ann. N.Y. Acad. Sci.* **256,** 289–303.
7. Robinson, D. R. & McGuire, M. B. (1975) *Ann. N.Y. Acad. Sci.* **256,** 318–329.
8. Robinson, D. R., Tashjian, A. H., Jr. & Levine, L. (1975) *J. Clin. Invest.* **56,** 1181–1188.
9. Goodson, J. M., Dewhirst, F. E. & Brunetti, A. (1974) *Prostaglandins* **6,** 81–85.
10. Wahl, L. M., Wahl, S. M., Mergenhagen, S. E. & Martin, G. R. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 3593–3601.
11. Wahl, L. M., Wahl, S. M., Mergenhagen, S. E. & Martin, G. R. (1975) *Science* **187,** 261–263.
12. Auletta, F. J., Zusman, R. M. & Caldwell, B. V. (1974) *Clin. Chem.* **20,** 1580–1587.
13. Levine, L., Hinkle, P. M., Voelkel, E. F. & Tashjian, A. H., Jr. (1972) *Biochem. Biophys. Res. Commun.* **47,** 888–896.
14. Bray, M. A., Gordon, D. & Morley, J. (1974) *Br. J. Pharmacol.* **52,** 453P.
15. Koopman, W. J., Gillis, M. H. & David, J. R. (1973) *J. Immunol.* **110,** 1609–1614.
16. Remold-O'Donnell, E. (1974) *J. Biol. Chem.* **249,** 3615–3621.
17. Gemsa, D., Steggemann, L., Menzel, J. & Till, G. (1975) *J. Immunol.* **114,** 1422–1424.
18. Dayer, J.-M., Krane, S. M., Russel, R. G. G. & Robinson, D. R. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 945–949.
19. Smith, J. W., Steiner, A. L. & Parker, C. W. (1971) *J. Clin. Invest.* **50,** 442–448.
20. Stockman, G. D. & Mumford, D. M. (1974) *Exp. Hematol.* **2,** 65–72.
21. Gordon, D., Bray, M. A. & Morley, J. (1976) *Nature* **262,** 401–402.