BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **225,** 34–39 (1996)
ARTICLE NO. 1127

A Possible Mechanism for the Stimulation of Metalloproteinase Production in Human Aortic Intimal Smooth Muscle Cells by Linoleic Acid Hydroperoxide

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Received June 27, 1996

To approach the mechanism of the stimulating effect of linoleic acid hydroperoxide on the production of matrix metalloproteinases (MMPs) in human aortic intimal smooth muscle cells (ISMC), we investigated the effect of the hydroperoxide on the cytosolic level of Ca^{2+}. Linoleic acid hydroperoxide provoked an increase in the cytosolic Ca^{2+} level, but it had no effect on the level of inositol phosphates (IPs) in these cells, in contrast with the effect of platelet-derived growth factor (PDGF), which elevated the level of both Ca^{2+} and IPs in these cells. A23187, a calcium ionophore, stimulated ISMC to produce matrix prometalloproteinase 1. These results indicate that linoleic acid hydroperoxide stimulates the production of MMPs in ISMC by elevation of the cytosolic Ca^{2+} level without the intervention of IPs. In addition, we found that the hydroperoxide has no effect on the binding of PDGF to its specific receptor on ISMC.

In a previous paper [1], we reported that linoleic acid hydroperoxide provokes injury to the aortic intima, and we considered that lipid peroxides increased in the blood initiate atherogenesis [2].

The tissue comprising the aortic intima consists not only of cells but also of an array of extracellular matrix macromolecules that support the skeleton of the cells and have a profound influence on their function. Therefore, extracellular matrix macromolecules would play an essential role in the reconstruction of the arterial intima after its injury. Regarding this problem, we reported previously that platelet-derived growth factor (PDGF) stimulates the production of matrix prometalloproteinase 1 (proMMP-1) in human aortic smooth muscle cells to affect the metabolism of collagen, which is a major protein among extracellular matrix components [3]. In a recent paper [4], we also reported that upon cultivation of human aortic intimal smooth muscle cells (ISMC) and endothelial cells in the presence of linoleic acid hydroperoxide, the production of matrix metalloproteinases (MMPs) in these cells was remarkably stimulated. This might accelerate the degradation of extracellular matrix macromolecules in the arterial intima during the process of atherogenesis.

Considering the importance of ISMC in atherogenesis [5], we decided to approach the mechanism of such stimulating effect of linoleic acid hydroperoxide on ISMC. It is known that binding of PDGF, the role of which is well known to be essential in atherogenesis, to its receptor rapidly stimulates cells to elicit various types of responses such as the activation of

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Abbreviations: MMPs, matrix metalloproteinases; ISMC, intimal smooth muscle cells; IPs, inositol phosphates; PDGF, platelet-derived growth factor; proMMP-1, matrix prometalloproteinase 1; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; IP_1, inositol monophosphate; IP_2, inositol diphosphate; IP_3, inositol triphosphate; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

0006-291X/96 $18.00

tyrosine kinase, hydrolysis of inositol phosphates (IPs), alteration of cellular pH, and an increase in cytosolic calcium level [6, 7]. It is also known that linoleic acid hydroperoxide elevates the level of Ca^{2+} in pig aortic endothelial cells [8]. On the basis of these facts, we investigated the effects of the hydroperoxide on the levels of cytosolic Ca^{2+} and IPs of ISMC in comparison with those of PDGF.

In relation to this study, we also carried out an experiment to determine whether the hydroperoxide has any effect on the binding of PDGF to its specific receptor on ISMC.

MATERIALS AND METHODS

ISMC. Aortic ISMC were isolated from human aortic intima and cultured as reported previously [9]. Briefly, after the thoracic organs including the aorta had been removed from a cadaver, the adventitia of the aorta was easily peeled off from one end. After removal of endothelial cells from the subendothelial bed by incubation in serum-free Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Tokyo) containing 1000 U/ml dispase (Sanko Pharmaceutical Co., Tokyo) for 90 min at 37°C, the inner surface was then washed with phosphate-buffered saline (PBS) to isolate ISMC from the intima. The ISMC were resuspended in DMEM supplemented with 10% fetal calf serum together with an antibiotic cocktail consisting of 100 μg/ml streptomycin, 100 U/ml penicillin, and 5 μg/ml fungizone, and cultured at 37°C under an atmosphere of humidified 5% CO_2-95% air mixture.

As aortic endothelial cells, we used the cells of the endothelial cell line SE_1 [10].

Linoleic acid hydroperoxide. Linoleic acid hydroperoxide was prepared enzymatically from linoleic acid, obtained from Sigma Chemical Co., St. Louis, MO, with soy bean lipoxygenase obtained from the same company, and purified by thin-layer chromatography with a solvent system of hexane-diethylether (8:2, v/v) as described previously [11]. The amount of the hydroperoxide was measured by the hemoglobin-methylene blue method [12]. Prior to use, the hydroperoxide was dissolved in ethanol to be 40 μmol/ml.

Other materials. PDGF used in the present experiment was PDGF-BB that was purchased from GIBCO, BRL, Grand Island, NY. Fura-2/AM was obtained from Wako Pure Chemical Industries Ltd., Osaka; [2-^{3}H]myo-inositol (10 Ci/mmol), from New England Nuclear, Boston, MA; and [^{125}I]-labelled PDGF-BB (1000 Ci/mmol), from Amersham, Buckinghamshire, UK. A23187, a calcium ionophore, was purchased from Sigma Chemical Co.

Measurement of Ca^{2+} level in single cells. The Ca^{2+} level in single cells was measured as reported previously [13]. Briefly, the cells were incubated for 30 min with fura-2/AM (1 μM) diluted with the culture medium and washed with the medium for 10 min. Further, the cells were washed twice with HEPES-buffered saline solution composed of 20 mM HEPES (pH 7.4), 115 mM NaCl, 5.4 mM KCl, 2.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 13.8 mM glucose. Then, the dish was placed on the thermostated stage of an Olympus IMT-2 inverted microscope. Agents dissolved in the above-mentioned HEPES-buffered saline solution were applied to the cells on a cover glass by the bath application method. The Ca^{2+} level in individual cells was measured fluorometrically. Fluorescence excitation was provided from a Hamamatsu 75W Xe lamp, and excitation wavelengths of 340 and 380 nm were selected by a computer-controlled movement of filters in the light path. Paired recordings were made every 5 sec, and the fluorescence images were obtained with a Hamamatsu SIT camera C2400-08 and stored in a digital image processor, Argus-100. The Ca^{2+} level was calculated on a pixel basis from the ratio of the fluorescence intensities obtained with excitation at 340 and 380 nm.

Measurement of [^{3}H]IPs. We followed the method described previously [13]. ISMC or endothelial cells cultured in 6-well culture plates (5×10^5 cells/well) were incubated with [^{3}H]inositol (1 μCi/well) at 37°C for 24 h. Reactions were started by the addition of linoleic acid hydroperoxide (100 nmol/ml) or PDGF-BB (10 ng/ml) in the presence of LiCl. After incubation for a definite time, 5% (w/v, final concentration) trichloroacetic acid solution was added to each well to terminate the reaction. Separation of [^{3}H]inositol monophosphate (IP_1), [^{3}H]inositol diphosphate (IP_2), and [^{3}H]inositol triphosphate (IP_3) was carried out by column chromatography with Bio-Rad AG1-X8 essentially as described by Berridge *et al.* [14]. Radioactivity in the elute was determined with a Packard Tri-Carb 2200 CA liquid scintillation counter.

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting analysis of proMMP-1. After ISMC had reached confluence in 35-mm Petri-dishes, 1 ml of serum-free fresh medium containing A23187 was added to the culture, and the cells were incubated for 2 days. After the incubation, the medium was taken and used for the analysis of proMMP-1. For immunoblotting, proteins of the culture medium (20 μl) separated by SDS-PAGE were electrotransferred to a nitrocellulose filter. After the filters had been washed with reconstituted non-fat dried milk (20%, w/v), they were treated with sheep anti-(human proMMP-1) serum (kindly donated by Dr. H. Nagase, Kansas Medical Center, Kansas City, KS) for 1 h. After having been extensively washed with PBS, the filters were incubated with peroxidase-conjugated rabbit anti-(sheep IgG) IgG for 1 h, and proMMP-1 was then visualized by staining of the peroxidase as described [3].

Assay for binding of PDGF. Assay for binding of PDGF was performed by the method of Heldin *et al.* [15]. ISMC cultured in 6-well culture plates (5×10^5 cells/well) were incubated in serum-free medium containing 1 μCi/well of

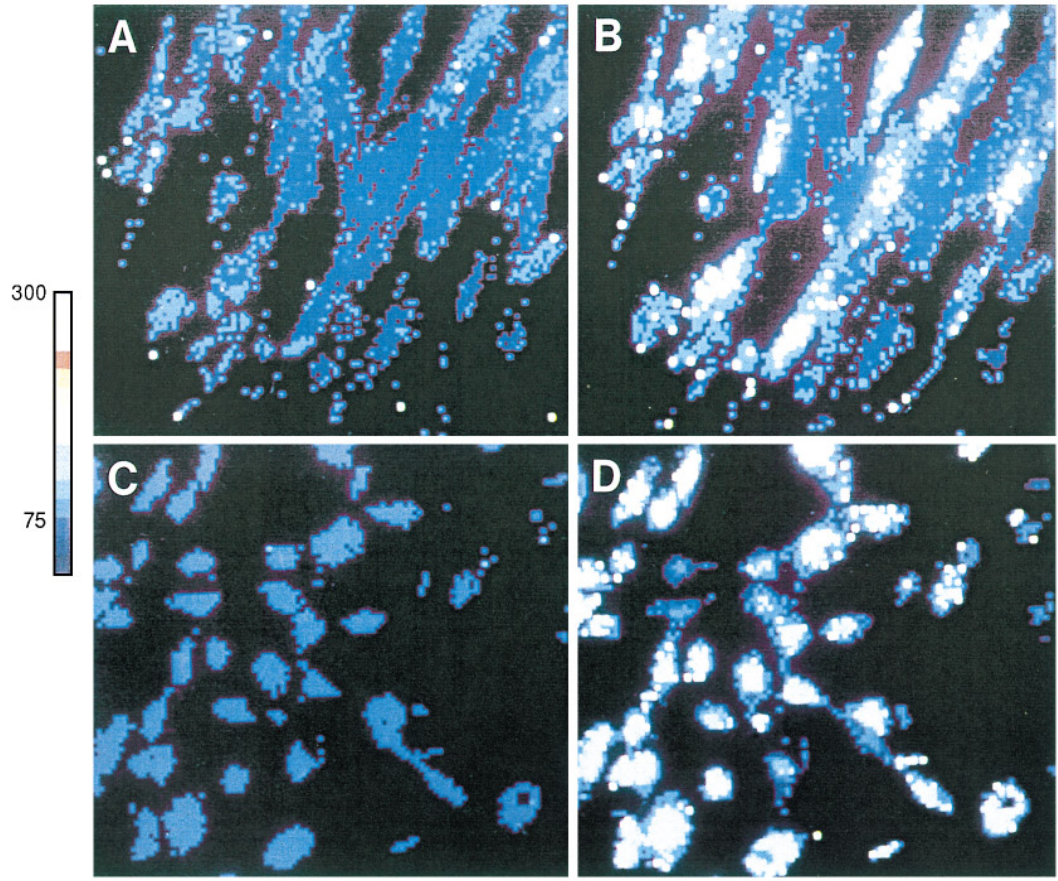

FIG. 1. Pseudocolor images of aortic ISMC and endothelial cells derived from the ratio of fura-2 fluorescence elicited by excitation at 340/380 nm after addition of linoleic acid hydroperoxide (final concentration, 100 nmol/ml). A: ISMC before the addition of the hydroperoxide; B: those after the addition of the hydroperoxide; C: Endothelial cells before the addition of the hydroperoxide; D: those after the addition of the hydroperoxide. Scale represents nM concentration of Ca^{2+}.

[^{125}I]-labelled PDGF-BB and 100 nmol/ml of linoleic acid hydroperoxide for different periods of time. After incubation, the medium was removed, and the cells were washed with PBS and mixed with ice-cold 10% (w/v) trichloroacetic acid solution. The sediment was dissolved in 10% SDS aqueous solution and was measured for radioactivity in a gamma counter.

RESULTS

Effect of linoleic acid hydroperoxide and PDGF on cytosolic Ca^{2+} level. By digital imaging fluorescence microscopy, we observed that the basal Ca^{2+} level in both aortic ISMC and endothelial cells was around 100 nM and that the Ca^{2+} was distributed uniformly in the cytoplasm. We found that 100 nmol/ml linoleic acid hydroperoxide induced an increase in the Ca^{2+} level in both aortic ISMC and endothelial cells incubated in the medium containing 2.2 mM $CaCl_2$ [Fig. 1]. In ISMC, the Ca^{2+} level was increased in the majority of them, although in the endothelial cells the elevation of the Ca^{2+} level was found in almost all cells.

When the time course of change in the Ca^{2+} level in individual ISMC was traced, the pattern of the response was different for different cells: some cells responsed immediately after the

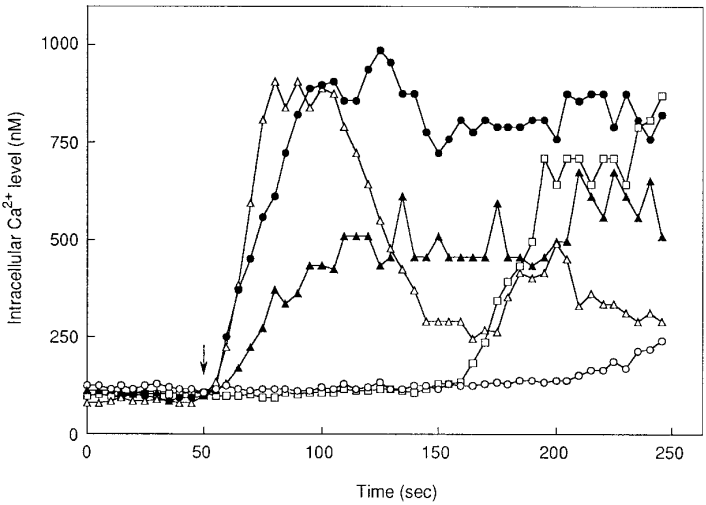

FIG. 2. Time course of change in the Ca^{2+} level of aortic ISMC in the presence of linoleic acid hydroperoxide. Each curve shows the time course of an individual cell. Arrow shows the time of addition of linoleic acid hydroperoxide (final concentration, 100 nmol/ml).

addition of the hydroperoxide to the culture, and others slowly. In Fig. 2, the patterns of Ca^{2+} elevation in response to linoleic acid hydroperoxide of five individual ISMC are shown.

When ISMC were stimulated with 10 ng/ml of PDGF-BB, all of the cells showed oscillatory Ca^{2+} elevation (Fig. 3). The cells showed individual variation in their time lag ranging from 15 to 35 sec. In the case of the endothelial cells, no such elevation was observed, as also shown in Fig. 3.

When calcium-free buffer was used, the elevation of the Ca^{2+} level mentioned above was not observed.

Effect of linoleic acid hydroperoxide and PDGF on IPs level. For this study, [^{3}H]inositol-

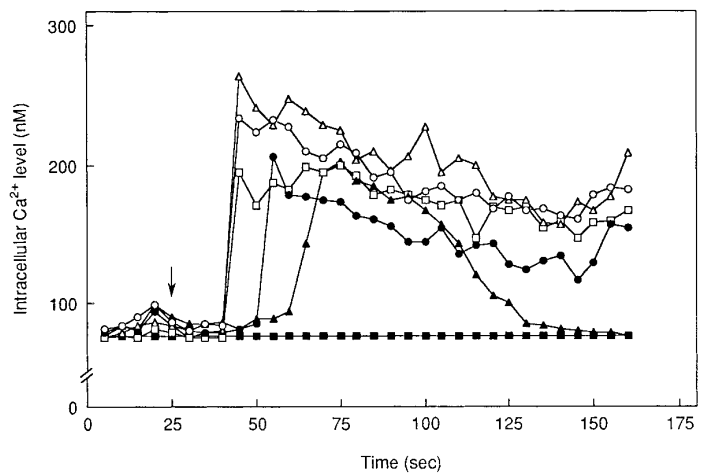

FIG. 3. Time course of change in the Ca^{2+} level of aortic ISMC and endothelial cells in the presence of PDGF. Each curve shows the time course of an individual cell. Arrow shows the time of addition of PDGF-BB. ○, ●, △, ▲, □: Ca^{2+} level in ISMC; ■, Ca^{2+} level in the endothelial cell.

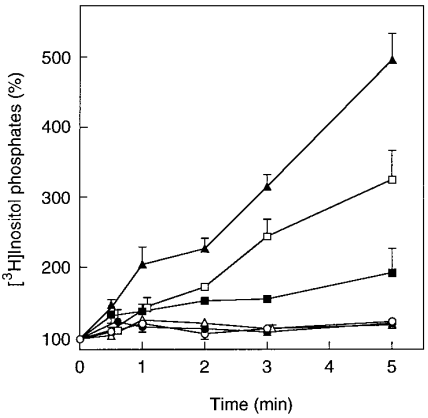

FIG. 4. Time course of [^{3}H]IPs accumulation in LiCl-treated human aortic ISMC. Linoleic acid hydroperoxide (100 nmol/ml) added: ○, IP_1; ●, IP_2; △, IP_3. PDGF-BB (10 ng/ml) added: ▲, IP_1; □, IP_2; ■, IP_3. Percentage of the control value is given. Mean ± SD. n=5.

labelled ISMC were incubated with linoleic acid hydroperoxide or PDGF-BB. Figure 4 shows the time course of [^{3}H]IP accumulation following the addition of 100 nmol/ml of the hydroperoxide or 10 ng/ml of PDGF-BB. The addition of linoleic acid hydroperoxide failed to increase [^{3}H]IP levels in ISMC. When PDGF-BB was added to the cell cultures, the amount of [^{3}H]IP_3 formed was 1.3 times greater than that of the control at as early as 30 sec after the addition, and it increased further with incubation time. The amount of [^{3}H]IP_1 accumulation was markedly increased at 30 sec after the addition, and reached a value 5 times over the control amount at 5 min after the addition. [^{3}H]IP_2 showed a medium accumulation.

The endothelial cells did not respond to either the hydroperoxide or PDGF in terms of increasing their levels of IPs (data not shown).

Effect of a calcium ionophore on proMMP-1 production. When 10^{-5} or 10^{-6} M A23187 (final concentration) was added to the cultures and incubation was carried out for 2 days, almost all of the cells died; whereas the addition of $10^{-7} \sim 10^{-9}$ M A23187 was not cytocidal, as judged light microscopically. Western blotting showed that the amount of proMMP-1 synthesized increased in the presence of $10^{-7} \sim 10^{-9}$ M A23187 as compared with the control amount, whereas no production of proMMP-1 was detected in the cells incubated with 10^{-5} or 10^{-6} M A23187 (Fig. 5). These results are consistent with those of microscopic observation.

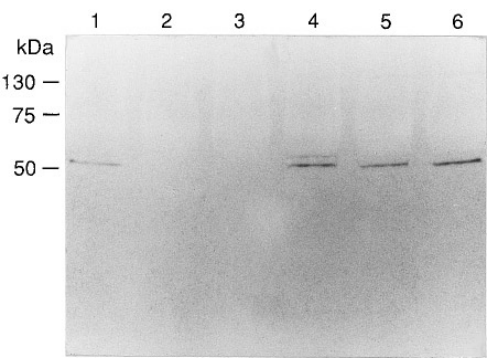

FIG. 5. Effect of A23187 on production of proMMP-1 in aortic ISMC. Lane 1, untreated cells. Lanes 2–6, cells treated with 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M A23187, respectively, for 2 days.

Effect of linoleic acid hydroperoxide on binding of PDGF to ISMC. Our experimental data showed that the presence of linoleic acid hydroperoxide (final concentration, 100 nmol/ml) had no effect on the binding of PDGF to its receptor on ISMC (data not shown).

DISCUSSION

In previous studies [3, 4], we found that both PDGF and linoleic acid hydroperoxide equally stimulated the production of MMPs which have an important implication in atherogenesis and other degenerative processes. Since PDGF is known to stimulate cells to elicit signal transduction that results in the elevation of the cellular Ca^{2+} level responsible for such stimulation, we suspected that linoleic acid hydroperoxide would also stimulate cells in a similar way.

On the basis of this consideration, we checked with ISMC whether linoleic acid hydroperoxide has any effects on the level of IPs and finally on the cellular level of Ca^{2+} in comparison with PDGF. The present results clearly demonstrated that the hydroperoxide elevated the cellular level of Ca^{2+} in aortic ISMC, although the response was different for different cells. In spite of such effect, the hydroperoxide had no effect on the metabolism of IPs. Thus, the mechanism of the elevation of the Ca^{2+} level in ISMC by the hydroperoxide is obviously different from that by PDGF. In addition, the hydroperoxide was found to have no effect on the binding of PDGF to its receptor.

Another of our present results showed that A23187, a calcium ionophore, strongly stimulated ISMC to produce proMMP-1. This finding indicates that a calcium flash from the extracellular fluid into the cytoplasm of the cell can stimulate the production of proMMP-1. Therefore, linoleic acid hydroperoxide is considered to stimulate the production of MMPs through the elevation of cellular Ca^{2+} without the intervention of IPs. It is also plausible that lipid hydroperoxides can influence, through elevation of the intracellular Ca^{2+} level, many processes other than MMPs production in the cells.

REFERENCES

1. Yagi, K., Ohkawa, H., Ohishi, N., Yamashita, M., and Nakashima, T. (1981) *J. Appl. Biochem.* **3,** 58–65.
2. Yagi, K. (1984) *BioEssays* **1,** 58–60.
3. Yanagi, H., Sasaguri, Y., Sugama, K., Morimatsu, M., and Nagase, H. (1991) *Atherosclerosis* **91,** 207–216.
4. Sasaguri, Y., Kakita, N., Murahashi, N., Kato, S., Hiraoka, K., Morimatsu, M., and Yagi, K. (1993) *Atherosclerosis* **100,** 189–196.
5. Ross, R. (1993) *Nature* **362,** 801–809.
6. Ross, R., Raines, E. W., and Bowen-Pope, D. F. (1986) *Cell* **46,** 155–169.
7. Kaibuchi, K., Tsuda, T., Kikuchi, A., Tanimoto, T., Yamashita, T., and Takai, Y. (1986) *J. Biol. Chem.* **261,** 1187–1192.
8. Hirosumi, J., Ouchi, Y., Watanabe, M., Kusunoki, J., Nakamura, T., and Orimo, H. (1988) *Biochem. Biophys. Res. Commun.* **152,** 301–307.
9. Murahashi, N., Sasaguri, Y., Ohuchida, M., Kakita, N., and Morimatsu, M. (1992) *Biotech. Appl. Biochem.* **16,** 152–159.
10. Sasaguri, Y., Yanagi, H., Nagase, H., Nakano, R., Fukuda, S., and Morimatsu, M. (1991) *Virchows Arch. B. Cell. Pathol.* **60,** 91–97.
11. Yagi, K., Ishida, N., Komura, S., Ohishi, N., Kusai, M., and Khono, M. (1992) *Biochem. Biophys. Res. Commun.* **183,** 945–951.
12. Yagi, K., Kiuchi, K., Saito, Y., Miike, M., Kayahara, N., Tatano, T., and Ohishi, N. (1986) *Biochem. Int.* **12,** 367–371.
13. Satoh, T., Sugama, K., Matsuo, A., Kato, S., Ito, S., Hatanaka, M., and Sasaguri, Y. (1994) *Atherosclerosis* **110,** 53–61.
14. Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P., and Irvine, R. F. (1983) *Biochem. J.* **212,** 473–477.
15. Heldin, C. H., Westermark, B., and Wasteson, Å. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78,** 3664–3668.