Biochemical Pharmacology, Vol. 35, No. 11, pp. 1879–1882, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

VERAPAMIL DEPRESSES THE SYNTHESIS OF LIPOXYGENASE PRODUCTS BY HYPOXIC CARDIAC RAT FIBROBLASTS IN CULTURE

BRIGITTE ESCOUBET,* GENEVIÈVE GRIFFATON and PAUL LECHAT
Institut de Pharmacologie, 15 rue de l'école de médecine, Paris, France

(*Received* 9 *October* 1985, *accepted* 21 *November* 1985)

Abstract—Lipoxygenase metabolites of arachidonic acid are potent chemotactic and vasoconstrictive agents and their local production in the myocardium induces the migration of polymorphonuclear cells into ischemic myocardium. These cells have been shown to play a role in the development of ischemic myocardial lesions. In the present study, the synthesis of arachidonic acid lipoxygenase metabolites by rat cardiac cells in culture and the effect of verapamil were investigated under normal and hypoxic conditions. Myocytes and fibroblasts metabolized exogenous arachidonic acid into 12-HETE and an unidentified metabolite (X). Fibroblasts synthesized significantly greater amounts of 12-HETE than myocytes ($P < 0.01$). Hypoxia (glucose-free medium and low PO_2) and verapamil (10^{-7} M) under normal conditions, did not change metabolite synthesis by either type of cells. Under hypoxia, verapamil decreased significantly 12-HETE and X production by fibroblasts ($P < 0.01$ and $P < 0.05$), whereas the synthesis in myocytes was not changed. It is concluded that the decrease in lipoxygenase product synthesis under hypoxia by verapamil may contribute to its therapeutic effects on the ischemic heart.

The metabolites of arachidonic acid, which are produced through the lipoxygenase pathways, exert various cardiovascular effects: leukotrienes, hydroperoxyeicosatetraenoic acids (HPETE) and hydroxytetraenoic acids (HETE) induce vasoconstriction, particularly constriction of the coronary vascular bed, and depression of the myocardial contractility [1, 2]. The metabolites are also chemotactic for polymorphonuclear leukocytes [3]. The white cell infiltration into the infarcted myocardium [4] could possibly be the consequence of the production of chemotactic agents by the infarcted heart [5]. Polymorphonuclear leukocytes can act on myocardium either by mechanical effects, by the occlusion of the microvascular bed [6, 7], or by biochemical effects, by the induction of inflammatory reaction and the release of toxic compounds [8]. The involvement of white cells in the development of myocardial ischemic lesions was suggested by the finding that the size of the infarcted area decreased when the number of circulating white cells was reduced [9, 10] or when the synthesis of lipoxygenase products was inhibited [11, 12]. However, the direct involvement of the lipoxygenase metabolites in the early stage of the development of myocardial infarction was not established.

Calcium antagonists are used for the prevention of angina pectoris [13] and for protection of the myocardium from ischemic injuries [14–16]. The effect of these drugs on the metabolism of arachidonic acid by the myocardium has not been investigated whereas, in the lung, nifedipine was shown to inhibit the release of the slow reactive substance of anaphylaxis, which is composed of a mixture of leukotrienes [17].

The purpose of the present study was to investigate the metabolism of exogenous arachidonic acid by lipoxygenase pathways in cultured cardiac cells and the influence of hypoxia and of verapamil upon this metabolism.

MATERIALS AND METHODS

Cell culture and identification of the cells. Myocytes and fibroblasts were obtained from 3-day-old Sprague-Dawley rates (Charles River, France). Ventricular cells were dissociated with porcine trypsin (Choay Labs, France) and myocytes were separated from fibroblasts by differential plating [8]. The cells were grown for 4 days in Minimal Essential Medium (MEM) (Eurobio, France) containing 10% new-born calf serum (Eurobio, France). Characterization of the cells, as myocytes and fibroblasts, was made by double indirect immunofluorescence using specific antibodies against myosin and tubulin [19]. This showed the purity of myocyte and fibroblast cultures, which contained at least 90% of the corresponding cell type when used [20].

Experimental procedure. On the fourth day of culture, the attached cells were washed 3 times with MEM and covered with 3 ml of protein and glucose-free MEM (Eurobio, France). The cells were gassed for 55 min through a rubber cap with air (20% O_2, 5% CO_2, 75% N_2) for the controls or with 95% N_2, 5% CO_2 for the cells submitted to hypoxia. Then, 0.5 μCi of ^{14}C-arachidonic acid (58 mCi/mmol, The Radiochemical Centre, Amersham, U.K.) was added to the MEM, in a 3 μl volume of ethanol, and

* Address for correspondence: Dr B. Escoubet, Faculté de Médecine X. Bichat, INSERM U 251, 16 rue H. Huchart, 75018 Paris, France.

the cells were further incubated for 1 hr in a rocking incubator at 37°. Verapamil (Knoll Pharmaceutical Co.) or BW755C (Wellcome Labs, U.K.) were dissoved in MEM and applied to the cells 5 min before the addition of the labeled arachidonic acid. Verapamil was used at a final concentration of 10^{-7} M. At the end of the incubation, the cells were washed 3 times with ice-cold 0.9% NaCl and scraped off on ice. An aliquot of the cell suspension was assayed for protein content [21] and the remaining cells were assayed for lipoxygenase products.

Lipoxygenase product assay. Cell suspensions were rapidly mixed with ice-cold methanol and, after acidification to pH 3, the supernatant was extracted twice with diethylether [22]. After evaporation to dryness, the extract was purified by silicic acid chromatography [22] and the fraction containing the lipoxygenase products used for separation by straight phase high performance liquid chromatography [23]. The separation was performed with a microporasil column (Waters Assoc., 250 mm long, 10 micrometre particle size) eluted with hexane:ethanol:acetic acid (98.7:1.2:0.1; v/v) at a flow rate of 1.0 ml/mm. Eluting products were continuously monitored by u.v. absorption at 235 nm wavelength. HETEs were identified by comigration with synthetic standards. The threshold of u.v. detection for 12-HETE was 5 ng. Fractions of the eluate were collected every 30 sec and counted for radioactivity by liquid scintillation (Packard M 4430, set for single label count and for sample quenching correction). The amount of HETE produced by the cells was estimated from the counted radioactivity, according to the specific activity of arachidonic acid, and expressed as ng/mg protein/hr or as pM/mg of protein/hr. Results were corrected for the recovery of the purification procedure estimated from the recovery of standards, and averaged 60%.

Statistical analysis. Comparisons were made by one way analysis of variance.

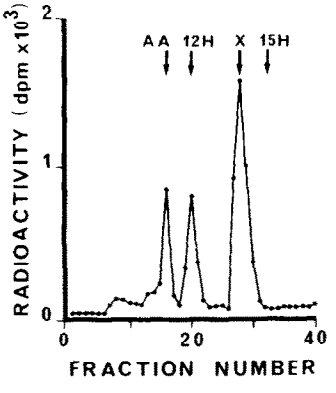

Fig. 1. Typical chromatogram of the lipoxygenase metabolites produced from exogenous arachidonic acid by myocardial cells in culture. Arrows indicate the migration of the corresponding standard as detected by u.v. (AA) arachidonic acid, (12H) 12-HETE, (X) unidentified product, (15H) 15-HETE. Eluting products were monitored up to 60 minutes and no significant other metabolite was detected.

RESULTS

Arachidonic acid metabolism by lipoxygenase pathway in cultured heart cells

The lipoxygenase metabolites produced from exogenous arachidonic acid by myocytes and fibroblasts were similar and are shown on the chromatographic profile (Fig. 1). Under basal conditions, the only identified metabolite was the 12-HETE. Another product (X) was detected which migrated between 12-HETE and 15-HETE. No C15- or C5-lipoxygenase metabolite was detected. Fibroblasts produced significantly greater amounts of 12-HETE than myocytes from exogenous arachidonic acid (Fig. 2). The production of lipoxygenase metabolites from endogenous arachidonic acid could be differently distributed in myocytes and in fibroblasts but it could not be assessed because the amount of unlabeled metabolites produced by a culture dish was small and did not reach the u.v. detection threshold.

Treatment of the cultures by the lipoxygenase inhibitor BW755C (40 mg/ml dissolved in medium) abolished the synthesis of both 12-HETE and the product X.

Effect of verapamil

In both type of cells, verapamil 10^{-7} M did not change 12-HETE and X production, as compared with controls (Tables 1 and 2).

Effect of hypoxic conditions

After the period of gassing with N_2 95% CO_2 5%, PO_2 in the incubation medium decreased from 121 ± 2.6 mmHg in controls to 24 ± 1.4 mmHg ($P < 0.001$) (mean ± S.E.M.). Under these conditions, the synthesis of 12-HETE and of X were not significantly changed in myocytes or in fibroblasts and no other metabolite was detected (Tables 1 and 2).

Effect of verapamil under hypoxic conditions

When verapamil was added to the cells after the gassing period, the amounts of 12-HETE and of X produced under hypoxia by fibroblasts was reduced

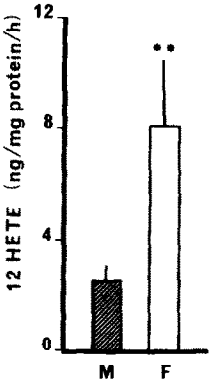

Fig. 2. Amounts of 12-HETE produced from exogenous arachidonic acid by rat cardiac myocytes and fibroblasts in culture. Fibroblasts (F) synthesize greater amounts of 12-HETE than myocytes (M) ($N = 12$). $^{**}P < 0.01$; results are shown as mean ± S.E.M.

Table 1. Synthesis of an unidentified metabolite (X) by rat cardiac cells in culture and effect of hypoxia and verapamil

	Air	Verapamil	Hypoxia	Hypoxia + Verapamil
Myocytes	15 ± 5	20 ± 6	16 ± 4	21 ± 12
Fibroblasts	46.3 ± 18	48 ± 8‡	42 ± 13†	10 ± 7*

Results are means ± S.E.M. and are expressed as pM/mg of protein/hr; N = 12; * $P < 0.05$, as compared with the control; † $P < 0.05$, ‡ $P < 0.01$ as compared with myocytes.

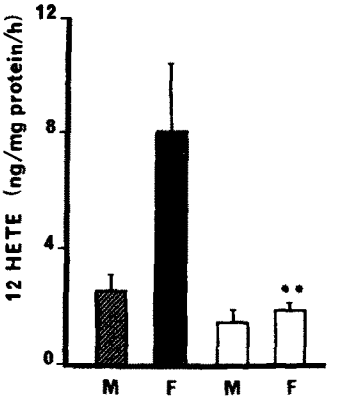

Fig. 3. Effect of verapamil (10 M) under hypoxic conditions on 12-HETE synthesis by cultured rat cardiac cells. (F) fibroblasts, (M) myocytes, open histograms are verapamil treated cells; (N = 12); **$P < 0.01$; results are shown as mean ± S.E.M.

significantly while, in myoctyes, it did not significantly change (Fig. 3 and Table 1).

DISCUSSION

The results show that rat myocardial cells metabolize exogenous arachidonic acid into 12-HETE and an unidentified metabolite. The synthesis of 12-HETE has been also shown in dog heart homogenates [24], whereas another group described the production of a leukotriene-like immunoreactive substance after stimulation by the calcium ionophore A 23187 of isolated perfused rat heart [25]. Other lipoxygenase pathways have been described in vascular tissues [26], as well as the release of leukotrienes from coronary arteries *in vitro* [27], so that vessels could account for part of the synthesis of lipoxygenase products by isolated heart and thus explain the discrepancies with our observations. *In vivo*, interactions with circulating white cells could explain the production of different metabolites [28]. In the chromatographic system used in the study, the X metabolite was found to migrate differently from the known HETEs. An undefined metabolite with similar chromatographic properties had also been found in cultured renal cells [29] but, as in our study the amounts synthesized by biological samples did not allow further chemical characterization. However, the inhibition of the synthesis of this product by the lipoxygenase inhibitor BW775C suggested that it was a lipoxygenase product.

Table 2. Effect of verapamil (10^{-7} M) and of hypoxia on 12-HETE synthesis by cardiac cells in culture

	Air	Verapamil	Hypoxia
Myocytes	2.5 ± 2	2.0 ± 1.6	1.8 ± 1
Fibroblasts	8 ± 6.6*	6.2 ± 2.8‡	7.5 ± 6.7†

Data are expressed as ng/mg of protein (mean ± S.D.); N = 12; *$P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ as compared with myocytes.

The reduction of the infarct size described after inhibition of the lipoxygenase pathways and also the increase in myocardial 12-HETE synthesis after *in vivo* ischemia [9, 24] suggested the direct involvement of lipoxygenase metabolites in myocardial ischemic injury [9, 30, 31]. In our study and in the isolated heart submitted to hypoxia [25] no change was detected in lipoxygenase metabolites of arachidonic acid. Difference in the experimental protocols, and particularly the use of models of *in vitro* ischemia, might account for this discrepancy, and suggest a role for circulating cells in the *in vivo* models of cardiac ischemia.

The important decrease observed in fibroblast 12-HETE synthesis (−78%) after treatment with verapamil under hypoxia was similar to that described in rat basophil cells [32]. A reduction of the production by fibroblasts of the chemotactic agent 12-HETE in the ischemic area could reduce, even if 12-HETE is not a very potent chemotactic agent, the initial migration of polymorphonuclear cells into the injured myocardium, as fibroblasts are the major producers of 12-HETE in myocardium. Polymorphonuclear leukocytes have been shown to cause microvascular plugging in infarcted heart [6, 7] and tissue injury through the release of toxic metabolites, such as leukotrienes [28], enzymes or free-radicals [33].

The mechanism involved in the action of verapamil upon the synthesis of lipoxygenase products cannot be directly addressed from this study. However, the synthesis of 12-HETE by fibroblasts from exogenous arachidonic acid was found depressed suggesting that the action of verapamil was exerted on the lipoxygenase enzyme itself. Similar findings were made in rat basophils with a depression of the 12-HETE production whereas the synthesis of PGE_2 was not reduced [32]. The small amounts of products synthesized from endogenous arachidonic acid precluded comparison of the effects of verapamil on the endogenous lipoxygenase metabolites. Nevertheless,

verapamil could exert effects on several cellular target points beside its effect on the entry of calcium through the calcium channels. This needs further investigations as well as the differences observed between myocytes and fibroblasts. The effects on the other pathways of the metabolism of arachidonic acid into prostaglandins are currently under investigation.

The changes in the myocardial metabolism of arachidonic acid induced by verapamil, particularly under hypoxia, might account for the protection by verapamil of myocardium against ischemic injuries. The balance of arachidonic acid metabolites during hypoxia is changed by verapamil towards protective agents either by increasing the synthesis of protective metabolites, such as prostacyclin [20], or by decreasing the production of chemotactic metabolites, such as 12-HETE. Moreover, the clinical relevance of these effects of verapamil was suggested by their occurrence at concentrations achieved during therapeutic use (10^{-8} to 10^{-7} M) [34].

Acknowledgements—The authors wish to thank Dr J. Maclouf and Dr J. Salmon for providing HETE standards and Knoll Laboratories for the supply of verapamil.

REFERENCES

1. J. A. Burke, R. Levi, Z. G. Gud and E. S. Corey, *J. Pharmac. exp. Ther.* **221**, 235 (1982).
2. F. Michelassi, L. Landa, L. Hill, W. D. Lowenstein-Watkins, A. J. Petkau and W. M. Zapol, *Science* **217**, 841 (1982).
3. S. R. Turner, J. A. Tainer and W. S. Lynn, *Nature, Lond.* **257**, 680 (1976)
4. W. Baumgartner, *Am. J. Physiol.* **2**, 243 (1899).
5. J. R. Hartmann, J. A. Robinson and R. M. Gunner, *Am. J. Cardiol.* **40**, 550 (1977).
6. M. D. Dahlgren, M. A. Peterson, R. L. Engler and G. W. Schmid-Schonbein, in *White Cell Mechanics: Basic Science and Clinical Aspects* (Ed. A. R. Liss), p. 271. Inc. 150 Fifth Avenue, New York (1984).
7. R. L. Engler, G. W. Schmid-Schonbein and R. S. Pavelec, *Am. J. Pathol.* **111**, 98 (1983).
8. P. Borgeat, B. Fruteau de Laclos and J. Maclouf, *Biochem. Pharmac.* **32**, 381 (1983).
9. K. M. Mullane and S. Moncada, *Prostaglandins* **24**, 255 (1982).
10. J. L. Romson, B. G. Hook, S. L. Kunkel, G. D. Abrams, M. A. Shork and B. R. Lucchesi, *Circulation* **67**, 1016 (1983).
11. G. A. Higgs, K. G. Mugridge, S. Moncada and J. R. Vane, *Proc. natn. Acad. Sci. U.S.A.* **81**, 2890 (1984).
12. J. L. Romson, B. G. Hook, V. H. Ricot, M. A. Schork, D. P. Swanson and B. R. Lucchesi, *Circulation* **66**, 1002 (1982).
13. M. B. Pine, P. D. Citron, D. J. Bailly, S. Butman, G. O. Plasencia, D. W. Landa and R. K. Wong, *Circulation* **65**, 17 (1982)..
14. M. M. Bershon and K. I. Shine, *J. Mol. Cell. Cardiol.* **15**, 659 (1983).
15. L. B. Rosenberger, L. W. Jacobs and H. C. Stanton, *Life Sci.* **34**, 1379 (1984).
16. I. Yamasawa, Y. Nohara, S. Konno, Y. Nagai, N. Yaoita, Y. Kaneko, H. Takahashi, M. Takanashi, T. Abe, T. Hachiya and H. Sassa, *Drug Res.* **33**, 389 (1983).
17. P. R. Butchers, J. F. Skidmore, L. J. Vardey and A. Wheeldom, *Br. Med. J.* **282**, 1792 (1981).
18. B. Blondel, I. Roijen and J. P. Cheneval, *Experientia* **27**, 356 (1971).
19. J. L. Samuel, B. Bertier, L. Bugaisky, F. Marotte, B. Swynghedauw, K. Schwartz and L. Rappaport, *Eur. J. Cell. Biol.* **34**, 300 (1984).
20. B. Escoubet, G. Griffaton, J. L. Samuel and P. Lechat submitted for publication.
21. O. H. Lowry, L. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
22. P. Borgeat, M. Hamberg and B. Samuelsson, *J. biol. chem.* **251**, 7816 (1976).
23. T. Winokur and A. R. Mottison, *J. biol. Chem.* **256**, 10221 (1981).
24. K. M. Mullane, N. Read, J. A. Salmon and S. Moncada, *J. Pharmac. exp. Ther.* **228**, 510 (1984).
25. M. Karmazyn and M. P. Moffat, *J. Moll. Cell. Cardiol.* **16**, 1071 (1984).
26. J. Larrue, M. Rigaud, G. Razaka, D. Daret, J. Demond-Henri and H. Bricaud, *Biochem. biophys. Res. Comm.* **112**, 242 (1983).
27. P. J. Piper, *Physiol. Rev.* **64**, 744 (1984).
28. J. Maclouf, B. Fruteau De Laclos and P. Borgeat, *Proc. natn. Acad. Sci. U.S.A.* **79**, 6042 (1982).
29. J. Sraer, M. Rigaud, M. Bens, H. Rabonovitch and R. Ardaillou, *J. biol. Chem.* **258**, 4325 (1983).
30. R. H. Bourgain, R. Andries, E. Finne, R. J. Vanderdriessche and P. J. Bernard, *Archs Int. Pharmacodyn.* **259**, 305 (1982).
31. S. Murphree, J. E. Saffitz, B. A. Jackschik and P. Needleman, *J. clin. Invest.* **75**, 992 (1985).
32. L. Levine, *Biochem. Pharmac.* **32**, 3023 (1983).
33. S. Z. Perkowski, A. M. Havill, J. T. Flynn and M. H. Gee, *Circ. Res.* **53**, 574 (1983).
34. M. D. McGoon, R. E. Ulietstra, D. R. Holmes and J. E. Osborn, *Mayo Clin. Proc.* **57**, 497 (1982).