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CYCLOOXYGENASE-DERIVED METABOLITES OF 8,9-EPOXYEICOSATRIENOIC ACID ARE POTENT MITOGENS FOR CULTURED RAT GLOMERULAR MESANGIAL CELLS

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Summary: The mitogenic effects of 11(R)-hydroxy-8,9-epoxyeicosatrienoic acid (EET) enantiomers were investigated in cultured rat glomerular mesangial cells. Both 11(R)-hydroxylated 8(R),9(S)- and 8(S),9(R)-EET at 1 μM stimulated [^{3}H]-thymidine incorporation to 300% and 280%, with 50% maximal effect occurring at $8x10^{-9}$ M and $1x10^{-8}$ M, respectively. Similar concentration-dependent effects were observed in stimulating induction of the immediate early gene, *c-fos*. Mitogenic activity of the 11(R)-hydroxylated enantiomers was not affected by prior downregulation of protein kinase C, suggesting involvement of protein kinase C-independent mechanisms. These findings suggest that either trans- or intracellular metabolism of 8,9-EET by cyclooxygenase occurs during inflammatory glomerular diseases and that the resulting metabolites are involved in mesangial cell proliferation.

Cytochrome P450 epoxygenase oxygenates arachidonic acid to form four regioisomeric epoxyeicosatrienoic acids (EETs)(1,2). Production of EETs was demonstrated originally in liver (3,4), and subsequently in medullary thick ascending limb of Henle of the rabbit kidney (5), platelets (6), vascular endothelial cells (7), and in the renal cortex of the rat (8) and of humans (9). In the kidney, EETs are thought to be involved in regulation of electrolyte and fluid reabsorption (10), glomerular hemodynamics (8,11), and inflammatory responses (12).

It has recently been recognized that P450 metabolites of arachidonic acid can be metabolized further by other arachidonic acid metabolic pathways, raising the possibility that the observed effects may be mediated by EETs modified in the target

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cells or adjacent cells. The vasoconstrictive effect of 8(S),9(R)-EET on renal hemodynamics was abolished in the presence of cyclooxygenase inhibition, suggesting that production of active metabolites by cyclooxygenase was required for the observed vasoconstriction (8,11). Our recent demonstration that cyclooxygenase of intact human platelets or ram seminal vesicles metabolized the enantiomers of 8,9-EET to corresponding 11(R)-hydroxylated enantiomers further suggests that the 11(R)-hydroxylated metabolites of 8,9-EET are the substance responsible for the observed effects (13).

We have previously demonstrated that 8,9-EET and 14,15-EET are mitogenic for cultured rat glomerular mesangial cells (12). In the present studies, we examined the effects of 11(R)-hydroxylated 8,9-EET enantiomers on mesangial cells and demonstrated that the 11(R)-hydroxylated metabolites were more potent mitogens than the parent 8,9-EET.

Materials and Methods

Cell culture Rat glomerular mesangial cells were cultured in RPMI 1640 containing 10 % fetal bovine serum and maintained as previously described (12). Studies were performed on cells between passages 21 to 32.

Preparation of 8,9-EET and 11(R)-hydroxylated metabolites 8,9-EET was prepared from arachidonic acid using standard procedures. Enantiomers of 8,9-EET were separated by a modification of our previously described HPLC method (14). The 11(R)-hydroxy-8,9-EET enantiomers were prepared from corresponding 8,9-EET enantiomers as we have previously described (13). Purified cyclooxygenase (400 µg) from ram seminal vesicles (15) was suspended in 2 ml of 0.1 M Tris-HCl (pH 8.0) containing 2 mM *p*-hydroxymercuribenzoate, 5 µM hemain, and 2 mM tryptophan. The suspension was bubbled with oxygen for 2 min and 8,9-EET enantiomer (100 µg, 156 µM) was added in 20 µl ethanol. The incubation was allowed to proceed for 5 min and stopped by the addition of 10 µl of citric acid. The incubation mixture was loaded on a C18 Sep-Pak, washed with 5 ml of water and products eluted with ethyl acetate. After evaporation of ethyl acetate, the residue was dissolved in 100 µl of acetonitrile, and purified by reverse phase HPLC on an Econosphere ODS column (4.6X250 mm, 5 µM; Alltech) using a linear gradient from water/acetonitrile/acetic acid (25/25/0.05, v/v/v) to acetonitrile/acetic acid (100/0.05, v/v) over 50 min at a flow rate of 1 ml/min. Absorbance was monitored at 235 nm. The 11(R)-hydroxy-8,9-EET enentiomers appeared at 26 min.

Determination of [^{3}H]thymidine incorporation Mitogenic effects were determined as previously described (12). Mesangial cells were plated at density of 3-5x10^5 cells/ml in media containing 10% fetal bovine serum. When cultures reached 70% confluency, the medium was switched to RPMI supplemented with 0.4% fetal bovine serum and incubated for 2 days prior to experiments. Agents to be tested were added to cultures and after 24 h of incubation, 1 µCi/ml [^{3}H]thymidine was added and incubation was continued for an additional 2 h. The reaction was stopped by washing cells with ice-cold phosphate buffered saline,

and 10% trichloroacetic acid precipitable label was collected as previously described (12). When indicated, protein kinase C was down-regulated by incubating cells with 0.1 μM phorbol 12-myristate 13-acetate (PMA) for 24 h prior to experiments (12).

Northern blotting Total RNA was isolated by the guanidine thiocyanate/phenol/chloroform extraction method (16). Total RNA of 15 μg was dissolved in 50% formamide and 2.2 M formaldehyde and electrophoresed in 1% agarose/2.2 M formaldehyde gel in 20 mM morpholinopropanesulfonic acid buffer. Samples were transferred overnight by capillary blotting in 10XSSC to nylon filters (Schleicher and Schuell, Keene, NH). The membranes were prehybridized at 65°C in 5XSSC, 5X Denhart's solution, 50 mM sodium phosphate, pH 6.5, 0.1%SDS, 250 μg/ml sonicated salmon sperm DNA, and 50% formamide. The membranes were hybridized overnight at 65°C in fresh hybridization solution in the presence of the ^{32}P-labeled cDNA probe and 10% dextran sulfate. The nylon membranes were washed with 2XSSC at room temperature for 15 min, followed by two washes in 0.2XSSC, 0.1% sodium dodecyl sulfate for 15 min at 65°C, and exposed to Kodak X-Omat X-ray films at -70°C with an intensifying screen. The cDNA probes used were *v-fos* (17) and β-actin (18) and were labeled to 10^8 cpm/μg by random priming procedure with [^{32}P]dCTP (DuPont New England Nuclear, Boston, MA) using a commercially available kit (GIBCO BRL, Gaithersburg, MD). The level of *c-fos* mRNA was quantitated by densitometry and normalized to that of β-actin.

Statistic analysis Results are expressed as means±SE. The Student's *t* test was used to evaluate the difference and $P<0.05$ was considered significant.

Results

Previously, we have demonstrated that 8,9-EET stimulated incorporation of [^{3}H]thymidine in mesangial cells, with maximal stimulation by 40% occurring at 1 μM (12). When tested under the similar conditions, both 11(R)-hydroxylated enantiomers of 8,9-EET were found to be more potent mitogens; at 1 μM, [^{3}H]thymidine incorporation was increased from 3,400±500 cpm/well in control to 10,100±900 and 9,400±800 cpm/well by 11(R)-hydroxylated 8(R),9(S)-EET and 8(S),9(R)-EET, respectively (Fig.1). Stimulation of [^{3}H]thymidine incorporation by the 11(R)-hydroxylated enantiomers was concentration-dependent, with 50% maximal effect achieved at 8×10^{-9} M and 1×10^{-8} M for 11(R)-hydroxy-8(R),9(S)-EET and 8(S),9(R)-EET, respectively (Fig.2).

A number of peptide mitogens induce cell proliferation in a manner dependent upon protein kinase C (19). The mitogenic effect of 11(R)-hydroxylated 8,9-EET enantiomers was not affected by prior downregulation of protein kinase C (Fig.3). In control cells, 11(R)-hydroxylated 8(R),9(S)-EET and 8(S),9(R)-EET stimulated [^{3}H]thymidine incorporation to 280±20% and 250±25% of that seen in non-stimulated cells, whereas in cells in which protein kinase C activity was down

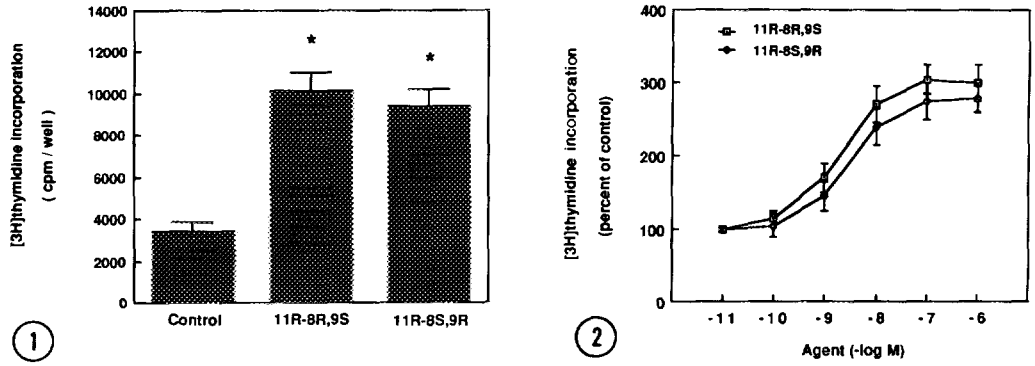

Fig.1. Stimulation of [^{3}H]thymidine incorporation in mesangial cells by 11(R)-hydroxy-8,9-EET enantiomers. Quiescent mesangial cells were incubated with 1μM of 11(R)-hydroxylated 8(R),9(S)-EET or 8(S),9(R)-EET for 24 h and incorporation of [^{3}H]thymidine was determined as described in *Materials and Methods*. Results of three independent experiments are shown. $^*P<0.05$.

Fig.2. Concentration-dependent stimulation of [^{3}H]thymidine incorporation by 11(R)-hydroxy-8,9-EET enantiomers. Quiescent mesangial cells were incubated with varying concentrations of 11(R)-hydroxylated 8,9-EET enantiomers for 24 h and incorporation of [^{3}H]thymidine was determined as described in *Materials and Methods*. Results of four independent experiments are shown as percent of values seen in control cells.

regulated by prior incubation of cells with 0.1 μM PMA, [^{3}H]thymidine incorporation was increased to a comparable degree, to 270±40% and 238±35% of that in non-stimulated cells by 11(R)-hydroxylated 8(R),9(S)-EET and 8(S),9(R)-EET, respectively.

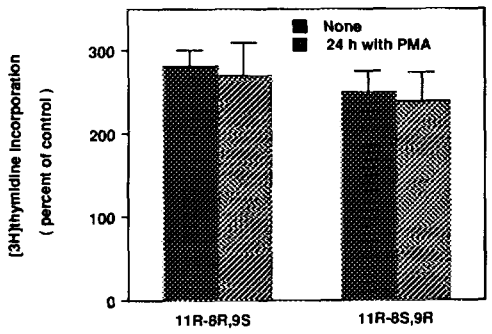

Fig.3. Effect of downregulation of protein kinase C on mitogenic effects of 11(R)-hydroxylated 8,9-EET enantiomers. Mesangial cells were incubated with or without 0.1 μM of PMA for 24 h. Cells were further incubated with 1 μM of 11(R)-hydroxylated 8,9-EET enantiomers and incorporation of [^{3}H]thymidine was determined. Results of three experiments are shown as percent of values seen in corresponding control cells.

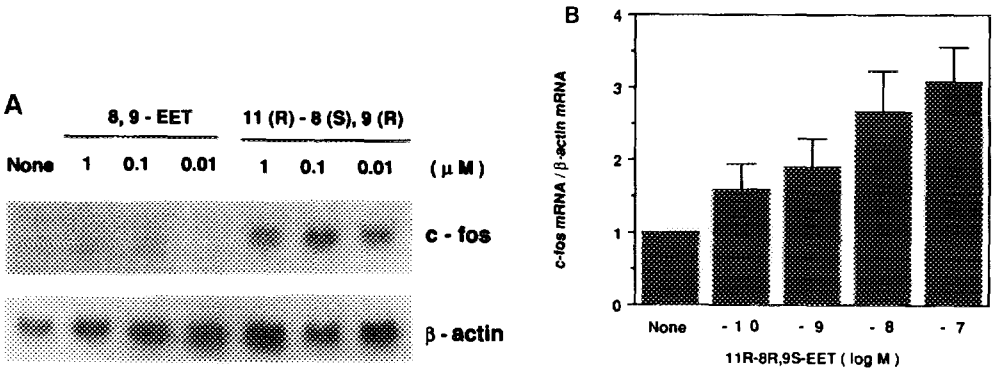

Fig.4. Induction of *c-fos* mRNA by 8,9-EET and 11(R)-hydroxy-8(S),9(R)-EET. Quiescent mesangial cells were incubated for 30 min with indicated concentrations of the indicated agent, total RNA was isolated, and 10 μg of total RNA was subjected to Northern blot analysis for *c-fos* mRNA and β-action mRNA as described in *Methods and Materials*. ***A***, Induction of *c-fos* mRNA by 8,9-EET and 11(R)-hydroxylated 8(S),9(R)-EET. ***B***, Concentration-dependent induction of *c-fos* mRNA by 11(R)- hydroxylated 8(S),9(R)-EET. The level of *c-fos* mRNA was quantitated by densitometry and normalized to that of β-actin.

The effect of 8,9-EET and 11(R)-hydroxy-8(S),9(R)-EET on the immediate early gene, *c-fos*, was determined by the Northern blotting analysis of total RNA (Fig.4). While *c-fos* mRNA was barely detectable with 1 μM of 8,9-EET, 11(R)-hydroxylated 8,9-EET at 0.01 μM was effective in stimulating expression of *c-fos* mRNA (Fig.4, A). The level of *c-fos* mRNA was increased by 11(R)-hydroxy-8(S),9(R)-EET, with concentration-dependence similar to that observed for stimulation of [^{3}H]thymidine incorporation (Fig.4, B).

Discussion

Previously, we have shown that 8,9-EET and 14,15-EET induced mitogenesis in mesangial cells (12). In the present studies, we demonstrated that hydroxylation of C-11 of 8,9-EET by cyclooxygenase resulted in generation of more potent mitogens for mesangial cells. In contrast to the parent 8,9-EET, which caused maximal stimulation of [^{3}H]thymidine incorporation by 40% (12), 11(R)-hydroxylated 8(R),9(S)-EET and 8(S),9(R)-EET stimulated [^{3}H]thymidine incorporation to 300% and 280%, respectively (Fig.1). In mesangial cells, 14,15-EET induced stimulation of [^{3}H]thymidine incorporation and activation of Na^+/H^+ exchange through protein kinase C-independent mechanisms (12). Similarly, prior down regulation of protein

kinase C by prolonged exposure of cells to PMA did not affect subsequent stimulation of [^{3}H]thymidine incorporation induced by the 11(R)-hydroxylated enantiomers (Fig.3), indicating that the11(R)-hydroxylated metabolites also exert mitogenic effects through protein kinase C-independent pathways.

Involvement of EETs in cell proliferation has also been demonstrated in primary cultures of rabbit proximal tubule cells (20). Mitogenic stimulation of proximal tubule cells by epidermal growth factor (EGF) has been shown to be accompanied by production of 5,6-EET and elevation in intracellular Ca^{2+} concentration due to increases in Ca^{2+} influx. Since 5,6-EET stimulated Ca^{2+} influx and the inhibitor of P450 monooxygenase, ketoconazole, abolished EGF-induced Ca^{2+} transient as well as mitogenesis, it was suggested that 5,6-EET acts as an intracellular second messenger mediating mitogenic response (20).

In the rat renal cortex, 8(S),9(R)-EET represents a major endogenous EET (11). Changes in the urinary excretion of EETs during pregnancy-induced hypertension has suggested a role for epoxygenase metabolites in the pathophysiology of human diseases (21). In the light of our previous demonstration that cyclooxygenase of platelets mediates hydroxylation of C-11 of 8,9-EET enantiomers (13), the present findings suggest that during inflammatory glomerular diseases, the cyclooxygenase of either infiltrating platelets or glomerular cells may mediate generation of 11(R)-hydroxylated 8,9-EET, and result in proliferation of mesangial cells.

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References

1. Capdevila, J.H., Falck, J.R., and Estabrook, R.W. (1992) FASEB J. 6:731-736.
2. Fitzpatrick, F.A., and Murphy, R.C. (1989) Pharmacol. Rev. 40: 229-241.
3. Capdevila, J., Chacos, N., Werringloer, J., Prough, R., and Estabrook, R. (1982) Proc. Natl. Acad. Sci. USA 78: 5362-5366.
4. Oliw, E., Guenguerich, P., and Oates, J. (1982) J. Biol. Chem. 257:3771-3787.
5. Ferreri, N., Schwartzman, M., Abraham, N., Chander, P., and McGiff, J. (1984) J. Pharmacol. Exp. Ther. 231:441-448.
6. Ballou, L.R., Lam, B.K., Wong, P.Y.-K., and Cheung, W.Y. (1987) Proc. Natl. Acad. Sci. USA 84: 6990-6994.
7. Abraham, N.G., Pinto, A., Mullane, K.M., Levere, R.D., and Spokas, E. (1985) Hypertension 7:899-904.

8. Takahashi, K., Capdevila, J., Karara, A., Falck, J., Jacobson, H.R., and Badr, K.F. (1990) Am. J. Physiol. 258:F781-F789.
9. Karara, A., Dishman, E., Jacobson, H., Falck, J.R., and Capdevila, J.H. (1990) FEBS Lett. 268:227-230.
10. Hirt,D.L., Capdevila, J., Falck, J.R., Breyer, M.D., and Jacobson, H.R. (1989) J. Clin. Invest. 84:1805-1812.
11. Katoh, T., Takahashi, K., Capdevila, J., Karara, A., Falck, J.R., Jacobson, H.R., and Badr, K.F. (1991) Am. J. Physiol. 261:F578-F586.
12. Harris, R.C., Homma, T., Jacobson, H.R., and Capdevila, J. (1990) J. Cell. Physiol. 144:429-437.
13. Zhang, J.Y., Prakash, C., Yamashita, K., and Blair, I.A. (1992) Biochem. Biophys. Res. Commun. 183:138-143.
14. Hammonds, T.D., Blair, I.A., Falck, J.R., and Capdevila, J.H. (1989) Anal. Biochem. 182, 300-303.
15. Marnett, L.J., Siedlik, P.H., Ochs, R.C., Pagels, W.R., Das, M., Honn, K.V., Warnock, R.H., Tainer, B.E., and Eling, T.E. (1984) Mol. Pharmacol. 26:328-335.
16. Chomczynski, P., and Sacci, N. (1987) Anal. Biochem. 162:156-159.
17. Curran, T., Peters, G., Beveren, C.V., Teich, N.M., and Verma, I.M. (1982) J. Virol. 44:674-682.
18. Cleveland, D.W., Lopata, M.A., MacDonald, R.J., Cowan, N.J., Rutter, W.J., and Kirschner, M.W. (1980) Cell 20:95-105.
19. Nishizuka, Y. (1988) Nature 334:661-665.
20. Burns, K.D., Homma, T., Karara, A., Capdevila, J., and Harris, R.C. (1991) J. Am. Soc. Nephrol. 2:426 (abstract).
21. Catella, F., Lawson, J.A., Fitzgerald, D.J., and FitzGerald, G.A. (1990) Proc. Natl. Acad. Sci. USA 87:5893-5897.