12(S)-Hydroxyeicosatetraenoic Acid Regulates DNA Synthesis and Protooncogene Expression Induced by Epidermal Growth Factor and Insulin in Rat Lens Epithelium

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Abstract
Neonatal rat lens epithelium has a high 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE] synthetic capacity, which decreases as epithelial cell proliferation decreases with age. To determine whether products of the 12-lipoxygenase pathway are involved in lens cell proliferation, we measured the effect of 12-lipoxygenase inhibitors on endogenous 12-HETE production, epidermal growth factor/insulin-stimulated DNA synthesis and protooncogene expression in cultured neonatal rat lens epithelial cells. Incubation of neonatal rat lenses in epidermal growth factor plus insulin, which stimulated endogenous 12-HETE production 8- to 10-fold, also produced a transient induction of c-fos and c-myc mRNAs after 2 to 3 h, followed by a round of DNA synthesis approximately 20 h later. The lipoxygenase inhibitor, cinnamyl-3,4-dihydroxy-α-cyanocinnamate, strongly inhibited both the endogenous 12-HETE synthesis and growth factor-stimulated DNA synthesis with a half-maximal inhibition between 10 and 20 μM. Cinnamyl-3,4-dihydroxy-α-cyanocinnamate (10 μM) also inhibited the expression of c-fos and c-myc mRNA and, to a lesser extent, c-jun mRNA. The inhibitory effects of cinnamyl-3,4-dihydroxy-α-cyanocinnamate on protooncogene expression and DNA synthesis were prevented by 0.3 μM 12(S)-HETE but not by equivalent concentrations of either 5(S)-HETE or 15(S)-HETE. These findings suggest that endogenously synthesized 12(S)-HETE may mediate epidermal growth factor/insulin-stimulated DNA synthesis in neonatal rat lens epithelial cells by regulating protooncogene expression.

Introduction
Various bioactive metabolites of arachidonic acid are implicated in the mitogenic response of cultured cells to stimulation by EGF.1 For example, the proliferative effect of EGF on mouse mammary epithelial cells is enhanced by the addition of prostaglandins or HETEs (1, 2). Prostaglandins mediate the EGF-induced increase in c-myc expression in rat hepatocytes (3). In EGF-treated mouse 3T3 fibroblasts, inhibitors of either the lipoxygenase or cyclooxygenase pathways of arachidonic acid metabolism partially block DNA synthesis (4, 5). Thus, evidence from several cell types suggests that arachidonic acid metabolites may be part of the signal transduction cascade initiated by EGF.

Among the earliest events in the proliferative response to EGF is a transient increase in expression of the protooncogenes, c-fos and c-myc (6–9). Induction of these genes is also seen when quiescent cultures are treated with serum or other growth factors (10–13) and seems to be required for cells to reenter the cell cycle (14–16) and progress to S (17). A large body of evidence supports the view that these protooncogenes code for transcription factors that regulate expression of genes required for cell cycle progression and DNA synthesis (12, 13, 18–20).

The lens presents an interesting model system in which to explore the relationship between EGF-induced mitogenesis, protooncogene expression, and arachidonic acid metabolites. EGF has been shown to induce proliferation in quiescent cultures of rabbit lens epithelial cells (21) and to support protein synthesis in organ-cultured neonatal rat lenses (22). Arachidonic acid metabolism by either the cyclooxygenase (23–25) or lipoxygenase (25–28) pathways has been demonstrated in the lenses of several species. In the neonatal rat lens, the principal lipoxygenase pathway product is 12(S)-HETE, as demonstrated by mass spectral analysis and chiral high performance liquid chromatography (27). The 12-LO activity in the epithelial cell layer of the rat lens, where all proliferative activity occurs (29), decreases sharply during the first 2 weeks after birth (27). Since a corresponding decrease in cell proliferation has been observed during this period (29), it is possible that 12-LO activity may be associated primarily with the proliferating cells of the lens epithelium in vivo.

To explore the possibility that 12-HETE may play a role in lens cell proliferation, we have examined the effect of lipoxygenase inhibitors and exogenous HETEs on DNA synthesis and protooncogene expression in neonatal rat lenses cultured in the presence of EGF and insulin. The results show that inhibition of endogenous 12-HETE synthesis decreases EGF-stimulated incorporation of 1H-thymidine into DNA. Sixteen to 20 h prior to the observed effect on DNA synthesis, expression of c-fos and c-myc mRNAs is strongly inhibited, while expression of c-jun mRNA is only slightly reduced. The effects of lipoxygenase inhibitors on protooncogene expression and DNA synthesis can be reversed by the addition of 12(S)-HETE but not by 5(S)-HETE or 15(S)-HETE, providing evidence for a novel regulatory pathway with a specific requirement for 12(S)-HETE. These findings suggest that endogenous synthesis of 12(S)-HETE by rat lens
epithelial cells may be part of a signal transduction mechanism activated by EGF, which, in the presence of insulin, leads to the mitogenic response.

**Results**

**Stimulation of Lens DNA and 12-HETE Synthesis by Insulin plus EGF.** Although EGF is known to stimulate DNA synthesis in rabbit lens epithelial cells (21), mitogenic activity toward rat lens epithelial cells has not been reported previously. Therefore, in preliminary experiments, we determined the ability of EGF to stimulate \[^{3}H\]thymidine incorporation into DNA in 4-day-old neonatal rat lenses in the presence and absence of insulin. An EGF-dependent increase in DNA synthesis was observed after approximately 20 h culture in the presence of insulin (Fig. 1). Neither EGF nor insulin alone increased DNA synthesis above levels observed in medium lacking growth factors (Fig. 2).

The addition of EGF plus insulin to the lens cultures also stimulated endogenous 12(S)-HETE production as measured by RIA from 5.2 ± 0.8 ng/ml in controls (media alone) to 40.5 ± 10 ng/ml in cultures containing growth factors (mean ± SE; n = 4–10). These results are in agreement with a previously published report of increased production of lipoxygenase metabolites after growth factor addition to BALB/c 3T3 cells (30).

**Effect of Lipoxygenase Inhibitors on DNA Synthesis.** The possible involvement of endogenous 12(S)-HETE synthesis in EGF-stimulated DNA synthesis was tested by measuring \[^{3}H\]thymidine incorporation into DNA in lenses cocultured in EGF, insulin, and the lipoxygenase inhibitor, CDC. These results were then compared with the production of radioimmunoassayable 12(S)-HETE in similarly treated samples. CDC inhibited both the \[^{3}H\]thymidine incorporation and endogenous 12(S)-HETE production in a dose-dependent manner with half-maximal inhibition for \[^{3}H\]thymidine and endogenous 12(S)-HETE at approximately 10 to 20 \(\mu\)M CDC (Fig. 3). The similarities in the dose response-curves is consistent with the possibility that inhibition of the lens 12-LO by CDC may be responsible for the decease in DNA synthesis.

To test whether the inhibition of endogenous 12(S)-HETE synthesis may be responsible for the decreased DNA synthesis, 0.3 \(\mu\)M 12(S)-HETE was added to the lens cultures in the presence of 10 \(\mu\)M CDC. This concentration of exogenous 12(S)-HETE was comparable to levels of endogenously synthesized 12(S)-HETE present in the medium after a 2-h incubation (40 ± 10 ng/ml, Fig. 3A). Exogenous 12(S)-HETE almost completely reversed the inhibitory effect of CDC on the \[^{3}H\]thymidine incorporation stimulated by EGF plus insulin (Fig. 4). Moreover, the requirement for 12(S)-HETE appeared to be specific, since neither 5(S)-HETE nor 15(S)-HETE reversed the effect of CDC (Fig. 4). Addition of 15(S)-, 12(S)-, or 5(S)-HETE to lens cultures in the absence of CDC did not significantly alter \[^{3}H\]thymidine incorporation (Fig. 4).

Curcumin, a mixed cyclooxygenase/lipoxygenase inhibitor (31) also inhibited \[^{3}H\]thymidine incorporation at concentrations that preferentially affect lipoxygenase activity (0.1 mm). As with CDC, the inhibition of DNA synthesis by curcumin was reversed by 0.3 \(\mu\)M 12(S)-HETE (Fig. 5).
Stimulation of c-fos and c-myc Expression by EGF. Measurement of c-fos and c-myc mRNAs in rat lens epithelial cells during the first few hours of stimulation by EGF and insulin showed a transient increase in expression of these proto-oncogenes (Fig. 6), similar to that observed in other cell types (6-9). Peak expression of c-fos occurred after about 2 h, followed by peak expression of c-myc at about 3 h. In subsequent experiments, both mRNAs were assayed at 2 h for convenience, although c-myc expression was not yet maximal.

Effect of Lipoxigenase Inhibitors on c-fos and c-myc Expression. To test whether products of the 12-LO pathway are involved in induction of c-fos and c-myc, lenses were incubated for 2 h in the presence of EGF and insulin with or without added CDC, and levels of c-fos and c-myc mRNA were determined by competitive PCR assay. The presence of 10 μM CDC strongly inhibited expression of c-fos and c-myc mRNA (Fig. 7, A and B). This result was obtained in three independent incubations with CDC and was also observed with two other lipoxigenase inhibitors, curcumin (0.1 mM) and nordihydroguaiaretic acid (20 μM; data not shown). Loss of these two mRNAs was not due to general loss of mRNA since levels of c-jun mRNA in the same samples were only slightly affected by CDC (Fig. 7C). Addition of 12(S)-HETE almost completely reversed the inhibitory effect of CDC on c-fos, c-myc, and c-jun expression (Fig. 8). Neither 15(S)-HETE nor 5(S)-HETE substituted for 12(S)-HETE, and addition of HETEs in the absence of CDC had little effect on protooncogene mRNA levels (Fig. 8).

Discussion

The present results suggest that the endogenous synthesis of 12(S)-HETE by neonatal rat lens epithelial cells plays a key role in the mitogenic response to EGF and insulin. This conclusion is supported by the following observations: (a) the addition of either curcumin or CDC, inhibitors of lens lipoxigenase activity, prevented the increased DNA synthesis normally elicited by EGF plus insulin; (b) the dose response curve for the ability of CDC to limit endogenous 12(S)-HETE production closely parallels the decreased lens DNA synthesis; and (c) addition of exogenous 12(S)-HETE or not 5(S) or 15(S)-HETE reversed the effect of the inhibitors on protooncogene expression and DNA synthesis. To our knowledge, this is the first report of a specific requirement for 12(S)-HETE in an EGF-stimulated response.

The data also suggests that the effect of lipoxigenase inhibitors on DNA synthesis may be due to their effects on protooncogene expression. The early induction of c-fos and c-myc protooncogene expression is strongly suppressed by inhibitors of the lipoxigenase pathway. c-jun was also affected to a lesser extent. Since c-jun transcription in human cells is known to be positively regulated by the c-fos/c-jun heterodimer (32), it is possible that the inhibition of c-jun expression observed in these experiments may be due to the loss of c-fos expression. Inhibiting expression of any of these protooncogenes has been shown to be sufficient to cause cell cycle arrest in G1 (14-17). Thus, the effect of lipoxigenase inhibitors on DNA synthesis in lens epithelial cells should be due to their effect on protooncogene expression.

Given the close association between cell proliferation and expression of c-fos and c-myc, it is not clear why lipoxigenase inhibitors produce only a 50% inhibition of EGF-stimulated DNA synthesis in rat lens epithelial cells when inhibition of c-fos and c-myc expression is almost total. Possibly some cells are able to express sufficient levels of c-fos and c-myc before inhibition of the lipoxigenase pathway occurs to permit cell cycle traversal and entry into S. Alternatively, following stimulation by EGF, some cells may be able to initiate DNA synthesis without expressing c-fos and c-myc.

EGF is only one of several growth factors that may regulate lens growth in vivo. Fibroblast growth factor is present in the anterior chamber of the eye (33, 34) and has been shown to stimulate DNA synthesis in neonatal rat lens epithelial explants (35) and bovine lens epithelial cells (36). Platelet-derived growth factor also stimulates DNA synthesis in bovine lens epithelial cells (and has been shown to support protein accumulation in the neonatal rat lens; Ref. 22). All of the above growth factors seem to act synergistically with the progression factors, insulin and insulin-like growth factor (21, 22, 37). The relationship of these other
growth factors to the 12-lipooxygenase pathway of the neonatal rat lens remains to be explored.

A growing body of evidence indicates that lipooxygenase pathway metabolites may regulate cell proliferation, although the mechanism is not clear and there may be differences in cell types (1, 2, 30, 31, 38–43). The present study demonstrates that the rat lens epithelium has a specific requirement for 12(S)-HETE as opposed to either 5(S)-HETE or 15(S)-HETE. Moreover, mass spectral analysis and chiral high performance liquid chromatography have shown that 12(S)-HETE is the principal lipooxygenase product synthesized by these cells (27).

Paradoxically, hydroxy-fatty acids have also been reported to inhibit DNA synthesis and cell division (44–47). These contradictory effects of HETEs on cell proliferation may also reflect cell type-specific responses. However, it should be noted that inhibitory responses have generally been observed at higher concentrations of HETEs (10 μM or greater) than are usually required for stimulatory responses. Since HETEs at these concentrations may inhibit lipooxygenase activity (47), inhibition of DNA synthesis by high concentrations of exogenous HETEs is not inconsistent with a stimulatory role for endogenously synthesized lipooxygenase products.

Fig. 6. Time course of induction of c-fos (A) and c-myc (B) mRNAs in neonatal rat lenses cultured in the presence of EGF plus insulin. mRNA levels were determined at the indicated times by coupled reverse transcription and PCR in the presence of internal standards.
In the neonatal rat lens, we have found evidence for a specific role for endogenous 12-HETE in EGF-stimulated DNA synthesis. This effect seems to be mediated by 12(S)-HETE-dependent protooncogene expression. The possibility that this EGF-stimulated pathway may be involved in regulating proliferation of other epithelial tissues raises intriguing clinical prospects for disorders involving epithelial hyperplastic conditions.

Materials and Methods

Lens Dissection and Culture. Lenses from 4-day-old Sprague-Dawley (Charles River) rats were removed and cleaned of any adhering iris with jewelers forceps and by gently rolling the lens on glassine paper. Lenses were incubated at 37°C in 5% CO₂ in 1 ml HL-1 media (Hyco Biomedical, Portland, ME) supplemented with 1 μg/ml insulin (Sigma Chemical Co.), 2 mM glutamine (GIBCO), 1 mM ascorbic acid, and 10 ng/ml mouse EGF (Boehringer-Mannheim). Lenses were incubated in either 24-well culture dishes for RNA analysis or 4-well multidishes (A/S Nunc, Roskilde, Denmark) for measurement of DNA synthesis by [³H]thymidine incorporation (see below).

Lenses were placed in the above medium with the following additions: (a) 0.1% ethanol (control); (b) 0.3 μM 12(S)-HETE, 5(S)-HETE, or 15(S)-HETE (Cyman Chemical, Ann Arbor, MI); (c) lipoxigenase inhibitor (10 μM CDC (Biomol, Plymouth Meeting, PA), 0.1 mM curcumin (Dr. Allan Conney, Rutgers University), or nordihydroguaiaretic acid (20 μM); and (d) 12(S)-HETE plus inhibitor.

Following timed incubations at 37°C, the lens capsule and the adhering epithelium were separated from the lens fiber mass by sweeping the tissue over a saline-saturated paper towel kept on ice. The capsule/epithelium was then collected with forceps for further assays.

Animals were maintained in accordance with the NIH Guidelines for the care and use of Animals (DWEW publication, NIH 80–2.3).

RIA of Rat Lens 12(S)-HETE. The media from rat lens cultures were assayed for 12(S)-HETE by RIA as described previously (27). The RIA was performed in a total volume of 0.2 ml containing [³H]12(S)-HETE (8,000 cpm) and a 1:2500 dilution of 12(S)-HETE antisera (generously provided by Dr. Lawrence Levine, Brandeis University). Unlabeled 12(S)-HETE standards (40–2000 pg) were run in each assay. After a 2.5-h incubation at room temperature, 0.5 ml of 0.9% NaCl containing 1% dextran and 1% charcoal was added, and the samples were immediately centrifuged at 2500 rpm at 4°C for 10 min to remove free unbound 12(S)-HETE. These assay conditions resulted in 50% bound 451 ± 24 pg 12(S)-HETE (mean ± SEM; n = 8).

[³H]Thymidine Incorporation. Neonatal rat lenses were cultured at 37°C for 0 and 24 h in the culture media described above with and without CDC and 12(S)-HETE. After incubation, the medium was removed, and the lenses were incubated for an additional h in Dulbecco's modified Eagle's medium supplemented with 1 μg/ml glutamine containing 10 μCi/ml [³H]thymidine (Amersham). The lenses were rinsed three times with cold Dulbecco's modified Eagle's medium, and the lens epithelium was gently dissected from the adhering fiber mass as described above. Incorporation of [³H]thymidine was determined by a modification of the method described by Funk and Sage (48). The tissues were kept overnight at 4°C in 0.25 ml 10% trichloroacetic acid containing 5% pyrophosphate and then centrifuged at 14,000 × g for 10 min at 4°C. The pellet was washed with 1 ml ethanol at -20°C, recentrifuged, decanted, and air dried. Fifty μl of 0.4 N NaOH was added, the tubes incubated at 60°C for 30 min, and the NaOH was neutralized with 0.5 ml glacial acetic acid. Ninety μl of the sample were transferred to a scintillation vial, and [³H]thymidine incorporation was determined by scintillation counting. The remaining sample was used for protein determination (49).

RNA Isolation. Lenses were incubated for 2 h, and the lens capsule and epithelial cell layer were separated from the lens fiber mass as described above. The capsule/epithelia from 3 to 5 neonates were pooled and homogenized in DEPC-washed glass homogenizers containing RNAzol B (Tel-Test, Friendswood, TX). RNA was isolated and collected by isopropanol precipitation and centrifugation fol-
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following the manufacturer’s protocol. The resulting pellet was dissolved in DEPC-treated water and reprecipitated with 0.8 M LiCl. The final RNA pellet was collected by centrifugation and dissolved in DEPC-treated water. RNA concentration was determined by absorbance at 260 nm.

Reverse transcription-PCR Assays. Oligonucleotides (20-mers) were synthesized for reverse transcription and PCR amplification (50, 51) of rat c-myc (52), c-fos (53), and c-jun mRNA (54). The regions amplified were: a 343-nt region of c-myc mRNA (737-1079) spanning the exon 2/exon 3 boundary; a 212-nt region of c-fos mRNA (1566–1777); and a 207-nt region of c-jun mRNA (2022–2228). The oligonucleotide sequences were checked for suitability using the PRIMER program (E. S. Lander, Whitehead Institute for Biomedical Research, Cambridge, MA), and oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer (Foster City, CA). The downstream oligonucleotide used for PCR amplification was also used for reverse transcription. Reactions were performed as described previously (55) using the magnesium optimum determined for each oligonucleotide pair (c-myc, 3 mM; c-fos, 3 mM; and c-jun, 5 mM).

For quantitation of relative mRNA levels, competitive PCR was performed with DNA internal standards (55, 56). The c-myc internal standard was synthesized using the method of overlap extension (57) to delete the region 916–987 yielding a 269-nt product. Internal standards for c-jun (120 nt) and c-fos (120 nt) were synthesized by using the overlap extension method to couple appropriate oligonucleotides to an available DNA fragment of known length. The c-jun internal standard (120 nt) was built upon a fragment of v-erbB DNA (Clonetech Mimic Kit; Clonetech Laboratories, Palo Alto, CA), while the c-fos internal standard (120 nt) was prepared using a fragment of chicken 6 crystallin DNA (58). In each case, the terminal 20 nt of the internal standard exactly matched the sequence of the oligonucleotides used for PCR so that each mRNA and its corresponding internal standard could be amplified simultaneously.

PCR assays used 100 ng of total cellular RNA per reaction with 10–50 fg of internal standard. Following PCR, the amplified products derived from the mRNAs and their internal standards were resolved on 8% polyacrylamide/urea sequencing gels (59). Products were detected by autoradiography, and scanning densitometry was used to quantitate the relative levels of PCR products. The ratio of the PCR product derived from the mRNA to that derived from the internal standard of each sample was normalized to the corresponding ratio in the control sample for each experiment. The use of normalized ratios corrects for possible differences in reverse transcriptase activity, gel loading, and autoradiographic conditions between experiments, thus allowing results of different experiments to be compared.

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