Pergamon

0006-2952(95)00217-0

Biochemical Pharmacology, Vol. 50, No. 7, pp. 947–952, 1995.

0006-2952/95 $9.50 + 0.00

DOWN-REGULATION OF EPIDERMAL GROWTH FACTOR-INDUCED 12-LIPOXYGENASE EXPRESSION BY GLUCOCORTICOIDS IN HUMAN EPIDERMOID CARCINOMA A431 CELLS

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(*Received* 13 *January* 1995; *accepted* 30 *March* 1995)

Abstract—The effect of glucocorticoids on epidermal growth factor (EGF)-induced expression of 12-lipoxygenase in human epidermoid carcinoma A431 cells was studied. A significant suppression of the EGF-induced expression of 12-lipoxygenase was observed in cells pretreated with 1 μM dexamethasone for 2 hr. The same pretreatment for 8 hr resulted in 55 and 54% inhibition of EGF-induced 12-lipoxygenase activity and mRNA expression, respectively. Cortisol, but not sex and mineral steroids, had a similar inhibitory effect. The glucocorticoid antagonist RU486 completely blocked the inhibitory effect of dexamethasone, suggesting that the action of dexamethasone was mediated through the ligation of glucocorticoid receptors. The results indicated that pretreatment of A431 cells with glucocorticoids resulted in a down-regulation of the EGF-induced expression of 12-lipoxygenase at the mRNA and enzyme activity level, which was mediated through glucocorticoid receptor activation.

Key words: 12-lipoxygenase; glucocorticoids; epidermal growth factor

Arachidonate 12-lipoxygenase (arachidonate:oxygen 12-oxidoreductase, EC 1.13.11.31) in platelets was the first mammalian lipoxygenase to be discovered [1, 2]. It catalyzes the transformation of arachidonic acid into 12(*S*)-hydroperoxyeicosatetraenoic acid, which is converted to 12(*S*)-HETE† by a glutathione-dependent peroxidase [3]. The enzyme was found subsequently in various animal tissues, including porcine [4] and bovine [5] leukocytes. However, the enzymes of different origins were found to be different in their substrate specificity. The leukocyte-type enzyme oxygenates not only arachidonic acid and other C_{20} acids but also C_{18} acids such as linoleic and linolenic acids, whereas the platelet-type enzyme is almost inactive with these C_{18} acids [6]. The cDNAs of human platelet/erythroleukemia cell (HEL) and porcine leukocyte 12-lipoxygenases, both of which have been cloned, share only 65% identity [7, 8]. Leukocyte-type 12-lipoxygenase was found recently in porcine anterior pituitary [9], bovine trachea [10], canine brain [11], and rat brain [12]. In addition to its presence in the platelets, the platelet-type enzyme was also identified recently in human epidermoid carcinoma A431 cells [13] and human skin epidermal cells [14].

In a previous study, we demonstrated the presence of a 12-lipoxygenase enzyme in a microsomal fraction of human epidermoid carcinoma A431 cells. The activity of 12-lipoxygenase was induced with a 10-hr lag period following treatment with an EGF [15]. We identified a human epidermal 12-lipoxygenase as the platelet-type enzyme by its substrate specificity, immunogenicity and RNA blot analysis, and showed that EGF up-regulated the expression of the 12-lipoxygenase mRNA [13]. This was the first evidence indicating the inducibility of 12-lipoxygenase expression by a growth factor, though an earlier study reported that phorbol 12-myristate 13-acetate induces the enzyme expression in human erythroleukemia cells [7, 16]. In studying the signal transduction leading to EGF-induced 12-lipoxygenase expression, we recently discovered that it involves the activation of protein kinase C [17]. In the present study, we used human epidermoid carcinoma A431 cells treated with EGF as a model of 12-lipoxygenase induction to demonstrate that dexamethasone down-regulated the EGF-induced expression of 12-lipoxygenase.

MATERIALS AND METHODS

Materials

Mouse EGF (natural, culture-grade) was purchased from Collaborative Research (Bedford, MA). [1-^{14}C]Arachidonic acid (56.3 mCi/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), multiprime DNA labeling system, rapid hybridization buffer, and nylon membrane (Hybond-N) were purchased from the Amersham Corp. (Bucks, U.K.). Dexamethasone, cortisol, progesterone, testosterone, estradiol and bovine serum albumin (fraction V) were obtained from Sigma (St. Louis, MO). RU486 was provided by Roussel-Uclaf (Romainville, France). Dulbecco's modified Eagle's medium and fetal bovine serum were from GIBCO BRL (Gaithersburg, MD) and HyClone (Logan, UT), respectively. Silica gel 60 thin-layer chromatography plates (0.25 mm thickness) were from E. Merck AG (Darmstadt, Germany). These and all other reagents used were of the highest purity available.

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† Abbreviations: COX, cyclooxygenase; EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRE, glucocorticoid-response element; and 12(*S*)-HETE, 12(*S*)-hydroxyiecosatetraenoic acid.

Cell culture EGF treatment

Human epidermoid carcinoma A431 cells were grown in 10-cm plastic dishes containing 10 mL of culture medium, consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 100 U/mL penicillin, in 5% CO_2 at 37°C. Confluent cells were subcultured from one dish to four dishes, and maintained without medium change until day 5 when they were treated with 50 ng/mL EGF dissolved in fresh culture medium. The medium for the control cells was also changed. After 30 min of EGF treatment, the medium was removed, and the cells were further cultured in fresh medium for up to 18 hr. In the steroid experiments, cells were pretreated with 1 μM steroid for 8 hr unless stated otherwise, and then treated with EGF as described above and incubated in the presence of steroid for up to 18 hr.

Preparation of microsomal fraction

A431 cells in a 10-cm petri dish were washed twice with phosphate-buffered saline and scraped with a Teflon sheet in 1 mL of 50 mM Tris–HCl, pH 7.4. About ten million cells were usually harvested from a 10-cm petri dish. Cells were then sonicated with a Heat Systems-Ultrasonics model W-375 sonicator. The homogenate was centrifuged at 9,000 *g* for 20 min, and the resulting supernatant was recentrifuged at 105,000 *g* for 1 hr in a Beckman L8-80 M ultracentrifuge. The resulting pellet was resuspended in 1 mL of 50 mM Tris–HCl, pH 7.4, and designated as the microsomal fraction. All the above procedures were performed at 4°. The protein content of microsomes was determined by the method of Lowry *et al.* [18] with bovine serum albumin (fraction V) as a standard.

Assay of microsomal 12-lipoxygenase activity

Microsomal 12-lipoxygenase activity was assayed as described [15]. One milliliter of the microsomal fraction containing 0.6 mg of protein was incubated with 3.3 μM [1-^{14}C]arachidonic acid (0.2 μCi) at 37° for 2 min. The reaction mixture was then acidified to pH 3 with 1 N HCl, extracted with 5 mL of ethyl acetate, and evaporated to dryness with nitrogen gas. Residues were dissolved in a small amount of absolute ethanol and applied to thin-layer chromatography plates. The plates were developed in the organic phase of a solvent system of ethyl acetate:2,2,4-trimethylpentane:acetic acid:water (11:5:2:10, volume ratio). The radioactive products were detected by autoradiography using Fuji X-ray (Rx medical) films. The zone corresponding to 12(*S*)-HETE was scraped into a scintillation vial, and its radioactivity was determined in a Pharmacia LKB Biotechnology Rackbeta liquid scintillation counter.

RNA preparation

Cells were washed with phosphate-buffered saline and scraped with a Teflon sheet. Cells from four dishes were harvested in 6 mL of Solution D composed of 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosinate and 0.1 M 2-mercaptoethanol. Total cellular RNA was isolated by an acid guanidium thiocyanate–phenol–chloroform method [19]. RNA content was determined by measuring the absorbance at 260 nm; about 500 μg of RNA was obtained from cells in four dishes. Intactness of RNA was examined on 1% agarose gels that were stained with 1 μg/mL ethidium bromide.

RNA blot analysis

Exactly 20 μg of total RNA was denatured with glyoxal, separated by electrophoresis on 1% agarose gels, and transferred to a nylon membrane as previously described [20]. The equivalency of samples was verified by the intensity of ethidium bromide staining of the 28 S and 18 S RNA bands and by hybridization with a probe for human GAPDH. The *NcoI-NcoI* fragment (940 base pairs) of human platelet 12-lipoxygenase cDNA [21] was used as a probe for the identification of 12-lipoxygenase mRNA in A431 cells. Probes were labeled with [α-^{32}P]dCTP by a Multiprime DNA-labeling system (Amersham), and hybridization with the ^{32}P-labeled probes was performed by a rapid hybridization system (Amersham). The nylon membranes were washed three times at room temperature in 2× SSPE (300 mM NaCl, 20 mM NaH_2PO_4 and 2 mM EDTA) containing 0.1% SDS, twice at 65° in 1× SSPE (150 mM NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA) containing 0.1% SDS, and finally twice at 65° in 0.5× SSPE (75 mM NaCl, 5 mM NaH_2PO_4 and 0.5 mM EDTA) containing 0.1% SDS, each time for 10 min. Then autoradiography was performed. The intensity of the hybridized band was determined by a Fujix Bio-imaging Analyzer BAS1000 (Fuji Photo Film Co., Tokyo, Japan), and the mRNA ratio of 12-lipoxygenase to GAPDH in each sample was obtained.

Statistical analysis

Data for 12-lipoxygenase activity and change in the relative percentage of mRNA expression were analyzed statistically by Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

EGF-induced expression of arachidonate 12-lipoxygenase in human epidermoid carcinoma A431 cells has been described earlier [13, 15]. An increased 12-lipoxygenase activity and a larger amount of 12-lipoxygenase mRNA accumulation were detected in cells treated with 50 ng/mL EGF for 18 hr compared with the untreated control. To study the regulatory effect of glucocorticoids on EGF-induced 12-lipoxygenase, cells were pretreated with 1 μM dexamethasone for 8 hr before EGF treatment. A typical RNA blot analysis is shown in Fig. 1. A 3.1-kb mRNA was detected in A431 cells as reported previously [13]. The induction of 12-lipoxygenase mRNA, which was detected in cells treated with EGF as described before, was suppressed by dexamethasone. Pretreatment with 1 μM dexamethasone resulted in 55 and 54% inhibition of EGF-induced 12-lipoxygenase activity and mRNA expression, respectively (Fig. 2). Under this experimental condition, the basal level of the enzyme activity and the mRNA expression in control cells also were inhibited slightly by dexamethasone. The inhibitory effect of dexamethasone was dependent on the length of preincubation with dexamethasone. As shown in Fig. 3, pretreatment with 1 μM dexamethasone for 2 hr significantly suppressed EGF-induced 12-lipoxygenase activity and mRNA expression, and the maximum inhibition was reached in cells pretreated with dexamethasone for 4 hr. Pretreatment for 1 hr had no signif-

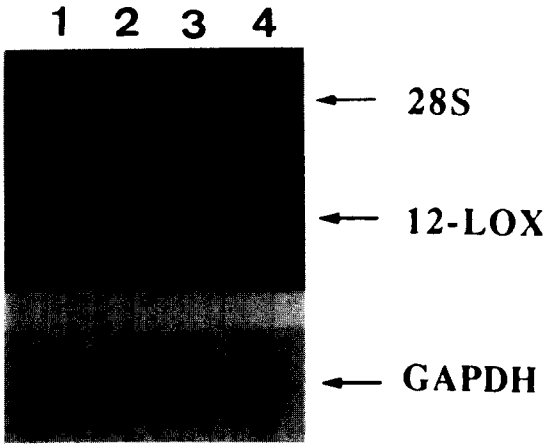

Fig. 1. RNA blot hybridization analysis of 12-lipoxygenase. Confluent cells were treated with 1 μM dexamethasone for 8 hr, followed by treatment with 50 ng/mL EGF for 30 min in the presence of dexamethasone. Dexamethasone was present in the culture medium in the ensuing 18-hr incubation after EGF treatment. Total RNA (20 μg) was separated by electrophoresis in 1% agarose. Lane 1, control; lane 2, dexamethasone; lane 3, dexamethasone plus EGF; and lane 4, EGF.

icant effect on EGF-induced 12-lipoxygenase expression. The inhibitory effect of dexamethasone was also concentration dependent, as shown in Fig. 4. Dexamethasone at concentrations of 0.1 and 1 μM resulted in 42 and 54% inhibition of EGF-induced 12-lipoxygenase mRNA expression, respectively; 0.01 μM dexamethasone had no significant effect.

The effects of several other steroids on EGF-induced expression of 12-lipoxygenase mRNA were also studied (Fig. 4). Cortisol, a physiological glucocorticoid, inhibited EGF-induced expression of 12-lipoxygenase mRNA. At 1 μM, cortisol resulted in 37% inhibition of enzyme induction, which was slightly weaker than the inhibition induced by 1 μM dexamethasone. Aldosterone, a mineralocorticoid, at 1 μM inhibited induction by 12%, which was statistically insignificant. Progesterone, testosterone and estradiol did not inhibit EGF-induced expression of 12-lipoxygenase mRNA.

RU486 is a noncompetitive antagonist of glucocorticoid receptors [22]. Hence, it was used to determine if the inhibition of dexamethasone on EGF-induced expression of 12-lipoxygenase was a receptor-mediated event. Cells were treated with 1 μM dexamethasone in either the presence or the absence of 1 μM RU486. The inhibition of EGF-induced expression of 12-lipoxygenase mRNA by dexamethasone was blocked completely by RU486. RU486 alone had no effect on the EGF-induced expression of 12-lipoxygenase (Fig. 5). In accord with the blocking effect on the inhibition of EGF-induced expression of 12-lipoxygenase mRNA by dexamethasone, RU486 also reversed completely the inhibition of EGF-induced expression of 12-lipoxygenase activity by dexamethasone (Fig. 6).

DISCUSSION

Fatty acid COX, lipoxygenase and cytochrome P450 epoxygenase are the three major pathways in arachidonate metabolism. Two isozymes of fatty acid COX have

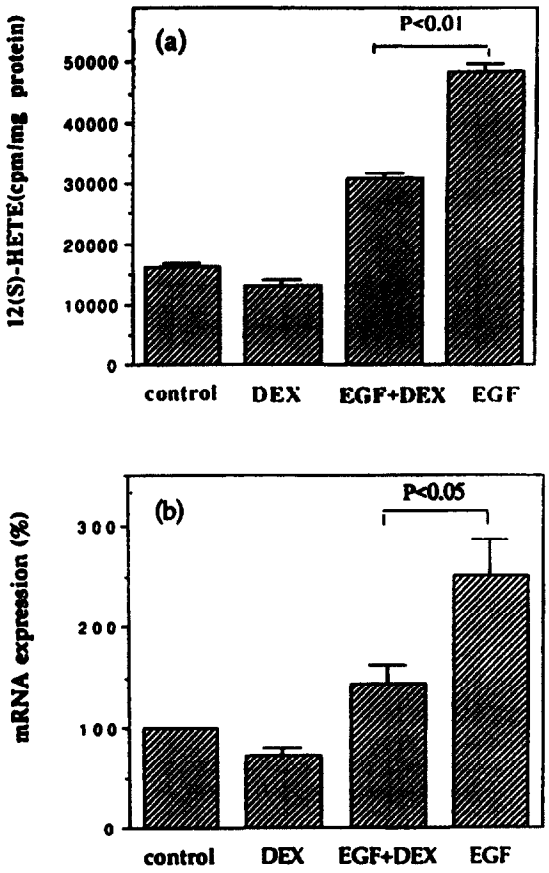

Fig. 2. Effect of dexamethasone on EGF-induced 12-lipoxygenase. Confluent cells were treated with 1 μM dexamethasone (DEX) for 8 hr, followed by treatment with 50 ng/mL EGF for 30 min in the presence of dexamethasone. Dexamethasone was present in the culture medium in the ensuing 18-hr incubation after EGF treatment, as described in Materials and Methods. 12-Lipoxygenase activity (a) and mRNA expression (b) in separate sets of cells were assayed after the 18-hr incubation. Values for enzyme activity are means ± SEM of 3 determinations, and those of mRNA are means ± SEM of 4 experiments. In the mRNA expression experiments, the intensity ratio of control cells was defined as 100%, and the relative intensity ratio of EGF and the dexamethasone-treated groups in each experiment was calculated.

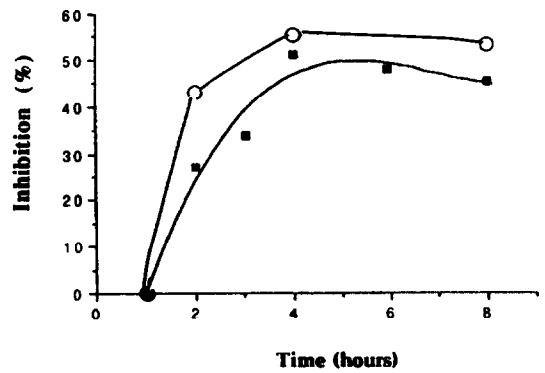

Fig. 3. Time-dependent effect of dexamethasone on EGF-induced 12-lipoxygenase. Confluent cells were pretreated with 1 μM dexamethasone for a certain period as indicated, followed by treatment with 50 ng/mL EGF. 12-Lipoxygenase activity (■) and mRNA expression (○) were assayed. Each value was the mean of two experiments, and the deviation was within 10%.

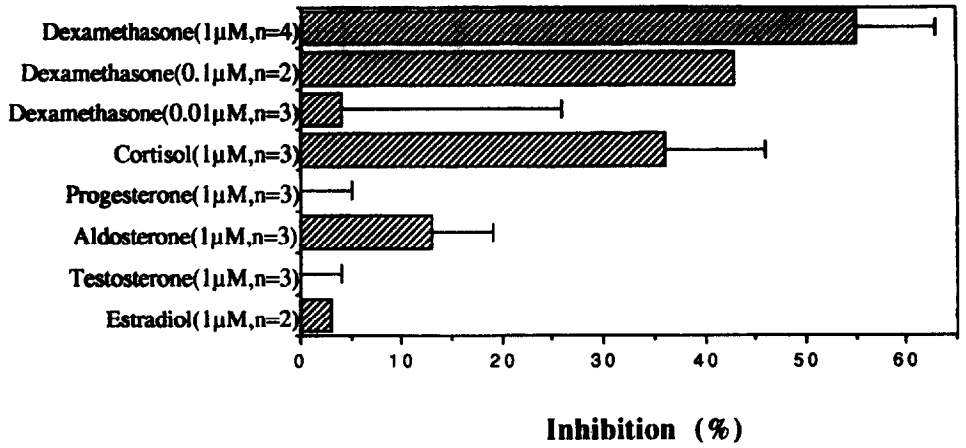

Fig. 4. Effects of steroids on EGF-induced 12-lipoxygenase mRNA expression. Confluent cells were pretreated with various steroids for 8 hr, followed by treatment with 50 ng/mL EGF. Values are means ± SEM. The number of experiments is indicated by n.

been identified. The first discovered form (COX-1) of COX encoded by a 2.8-kb mRNA is considered to be a constitutive enzyme [23], and a mitogen-inducible form (COX-2) of the enzyme encoded by a 4-kb mRNA is considered to play an important role in inflammation [24]. Glucocorticoids strongly inhibit the expression of the 4-kb mRNA of COX-2, but do not change the expression of the 2.8-kb mRNA of COX-1 [25].

The regulation of lipoxygenase pathways is less well understood than that of fatty acid COX. Conrad *et al.* [26] found that interleukin 4 up-regulates the 15-lipoxygenase expression in human monocytes, and treatment with 10^{-7} M hydrocortisone inhibits the interleukin 4-induced 15-lipoxygenase expression. EGF up-regulates the 12-lipoxygenase expression in A431 cells [13]. In this study, we found that pretreatment of A431 cells with dexamethasone inhibited the EGF-induced expression of 12-lipoxygenase in a concentration-dependent manner (Fig. 4). The inhibitory effect was glucocorticoid-specific because while cortisol was also effective, the nonglucocorticoids such as progesterone, aldosterone, testosterone and estradiol had no significant effect (Fig. 4). Further evidence that the action of dexamethasone was mediated through the ligation of corticosteroid receptors was provided by experiments utilizing the steroid antagonist RU486. RU486 stabilizes the association of steroid receptors with heat shock protein 90 in the presence of ligand, which prevents translocation of glucocorticoid receptors to the nucleus and thereby blocks transcription of genes containing GREs [22, 27]. RU486 treatment completely reversed the inhibition of EGF-induced expression of 12-lipoxygenase in A431 cells by dexamethasone (Figs. 5 and 6). Since progesterone was not effective in inhibiting the EGF-induced expression of 12-lipoxygenase, the complete reversal by RU486 indicates that the inhibition of dexamethasone on EGF-induced expression of 12-lipoxygenase was mediated through glucocorticoid receptors. 12(*S*)-HETE may play

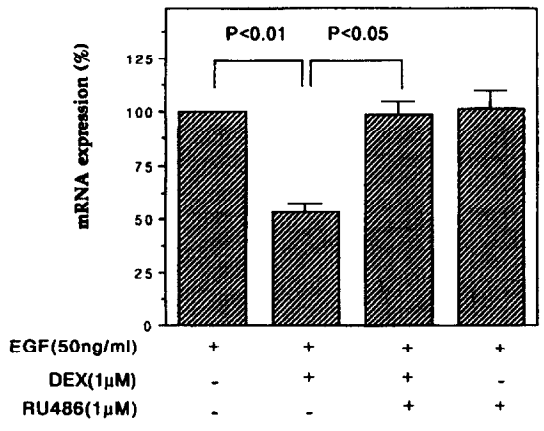

Fig. 5. Effect of RU486 on the inhibitory action of dexamethasone. Confluent cells were treated with 1 μM dexamethasone in the presence or absence of 1 μM RU486 for 8 hr, followed by treatment with 50 ng/mL EGF. In the RU486-treated cells, 1 μM RU486 was present in the culture medium until RNA extraction. Values are means ± SEM of 3 experiments. Calculation of mRNA expression was performed in the same manner as described in the legend of Fig. 1.

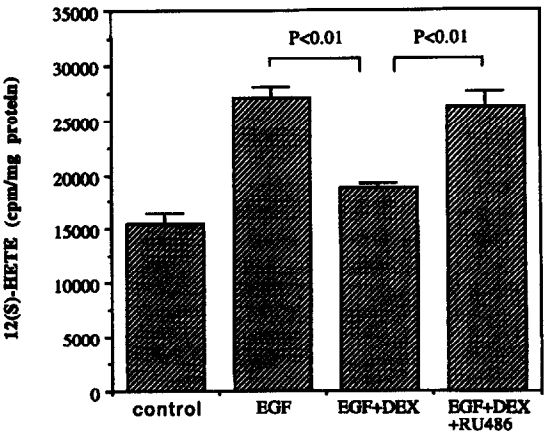

Fig. 6. Effect of RU486 on the inhibitory action of dexamethasone at the enzyme activity level. Confluent cells were treated with 1 μM dexamethasone in the presence or absence of 1 μM RU486 for 8 hr, followed by treatment with 50 ng/mL EGF. 12-Lipoxygenase activity was assayed after an 18-hr incubation. Values for enzyme activity are means ± SEM of 4 determinations.

a significant role in the pathogenesis of some epidermal and epithelial inflammation, since (a) the topical or intradermal administration of 12-HETE may cause epidermal inflammation and proliferation [28], and (b) the expression of 12-lipoxygenase is induced significantly in the epidermis of patients with inflammatory psoriasis [29, 30] and in colonic tissues from patients with inflammatory bowel disease [31]. Hussain *et al.* [30] recently identified the 12-lipoxygenase that is overexpressed in germinal layer keratinocytes in psoriasis as a human platelet-type enzyme. The present result indicating the down-regulation of the expression of platelet-type 12-lipoxygenase by dexamethasone provides a pharmacological basis for the anti-inflammatory effects of glucocorticoids in human epidermal psoriasis.

A gene encoding human platelet-type 12-lipoxygenase has been isolated, and an approximately 1-kb sequence of the 5′-flanking region of the translation initiation site has been determined [32, 33]. The 5′-flanking region contains several regulatory elements including four GC boxes, two CACCC boxes, three AP-2 binding sequences, and a GRE. The transcriptional gene regulation of GREs can be either positive or negative, depending on the machinery of transcription factors involved in the cell. Diamond *et al.* [34] proposed a composite GRE action model that invokes both DNA binding and protein–protein interactions by receptor and nonreceptor factor, and this model may be the mechanism responsible for the negative regulation of the collagenase promoter [35, 36] and proliferin promoter [34] by glucocorticoids. It is still not clear whether the GRE in the 5′-flanking region of 12-lipoxygenase gene mediates the inhibitory action of dexamethasone on EGF-induced expression of 12-lipoxygenase mRNA. Study on the promoter regulation with GRE-deleted plasmid constructs is underway.

Acknowledgements—We are greatly indebted to Drs. Tanihiro Yoshimoto and Shozo Yamamoto for providing cDNA of human platelet 12-lipoxygenase and to Dr. Lee-Young Chau for providing cDNA of human GAPDH. We also thank Roussel-Uclaf (Romainville, France) for providing RU486. The excellent technical assistance of Y. S. Wen and R. S. Jong is appreciated. Thanks are also due to Drs. Shen K. Yang, Kun-yen Huang and George S. Roth for their critical review of this manuscript and to Y. J. Chang for her secretarial assistance. This work was supported, in part, by Grant NSC83-0412-B006-050 from the National Science Council of the Republic of China.

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