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SHORT COMMUNICATION

Role of Leukocyte Influx in Tissue Prostaglandin H Synthase-2 Overexpression Induced by Phorbol Ester and Arachidonic Acid in Skin

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ABSTRACT. The accumulation of neutrophils and mononuclear cells is a characteristic feature of 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced ear edema. This cell influx was accompanied by the enhancement of eicosanoid tissue levels and prostaglandin H synthase-2 (PGHS-2) overexpression. Sialidase treatment, which affects the structure of selectins and inhibits leukocyte influx, significantly reduced eicosanoid and PGHS-2 levels and edema. In contrast, skin PGHS-2 overexpression induced by arachidonic acid (AA) application was not affected by sialidase treatment. These results suggest that PGHS-2 overexpression induced by TPA could be induced by AA and/or AA metabolite release by leukocyte infiltrated during the inflammatory process. BIOCHEM PHARMACOL **58**;5:877–879, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. arachidonic acid; cyclooxygenase; acute inflammation; prostaglandins; leukotrienes; anti-inflammatory drugs

Prostaglandin H synthase (PGHS†; EC 1.14.99.1), also called cyclooxygenase, catalyzes the production of prostaglandins. Two isoforms of PGHS have been characterized: PGHS-1 is considered to be a constitutively expressed housekeeping gene, whereas PGHS-2, an immediate early gene, appears to be expressed only by specific stimuli such as mitogens and cytokines [1–3], while being inhibited by anti-inflammatory steroids [4]. These data suggest that PGHS-2 may contribute to the development of the inflammatory process. Thus, PGHS-2 has been detected in the inflammatory site of models such as carrageenin-induced pleurisy [5, 6], air pouch [7], and paw edema [8] in rats. Moreover, the expression of PGHS-2 is increased in synovia from patients with rheumatoid arthritis [9]. These observations, which show that this cyclooxygenase isoform is detectable in inflammatory sites *in vivo*, indicate a role for this enzyme in the development of the inflammatory response.

Recently, we observed PGHS-2 overexpression correlated with the time–course of TPA- and AA-induced edema formation in skin. These results suggested that AA release and/or subsequent metabolism by the PGHS pathway may be involved in the induction of PGHS-2 expression in murine TPA- and AA-induced ear edema [10]. Using these experimental models of inflammation, we have previously shown [11] that prostanoid levels increase at an early stage of edema, and we suggested that these prostanoids may play an important role in cell migration and plasma exudation. The purpose of this paper was to study the contribution of leukocyte influx to PGHS-2 overexpression during the development of these acute inflammatory processes.

MATERIALS AND METHODS

Materials

AA, TPA, *o*-dianisidine 2HCl, PMSF, aprotinin, leupeptin, diethyldithiocarbamic acid, and sialidase were obtained from Sigma Chemical Co. Human polymorphonuclear leukocyte MPO and NAG from beef kidney and *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside were obtained from Calbiochem. Ovine PGHS-2 and rabbit polyclonal antisera were obtained from Cayman Chemicals Co. All other reagents were of analytical grade.

AA and TPA Ear Inflammation Models

TPA or AA was dissolved in acetone and 20 μL of solution was applied to the inner and outer surface of the right ear of Swiss Webster mice (Interfauna). The left ear received acetone, delivered in the same manner. Finally, the mice were killed by CO_2 inhalation, a 7-mm diameter section of the right and left auditory pinna, measured from the apex, was cut, and the samples were weighed and used for PGHS determinations. Ear edema was measured as the difference

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† *Abbreviations:* AA, arachidonic acid; PGHS-2, prostaglandin H synthase-2; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PMSF, phenylmethylsulfonyl fluoride; MPO, myeloperoxidase; and NAG, *N*-acetyl-β-D-glucosaminidase.

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in weight between the challenged and the unchallenged ear, and expressed as percentage.

Measurements of MPO and NAG

MPO, a hemoprotein located in the azurophil granules of neutrophils, was used as an enzyme marker of neutrophil infiltration into inflamed tissues, as proposed by Bradley *et al*. [12]. Similarly, NAG levels were used as an indicator of mononuclear cell infiltration [13]. MPO and NAG assays were performed according to procedures used previously [11].

Measurements of Eicosanoid Metabolites

Mouse ears were homogenized with 1 mL of methanol containing 1% 1 M HCl. Two mL of distilled water was then added to each tube, and the tubes were kept on ice for 30 min. The resulting insoluble proteins were removed by centrifugation (30,000 *g*, 20 min) and prostaglandin E_2 (PGE_2), 6-keto-prostaglandin $F_{1\alpha}$, and leukotriene B_4 (LTB_4) were measured as previously described [11], using the specific protocols set out for EIA kits from Cayman Chemicals Co.

Protein Determination and Western Blot Analysis

Mouse ears were homogenized with 0.5 mL of PBS containing 2 mM EDTA, 250 μg/mL PMSF, 250 μg/mL aprotinin, 250 μg/mL leupeptin, and 200 μg/mL dimethyldithiocarbamic acid, and lysates were normalized using the BioRad protein assay kit and stored at −80° until use. Bromophenol blue (0.05% w/v) and 2-mercaptoethanol (6% v/v) were added to equal amounts of protein (20 μg) and the mixture was boiled for 10 min. SDS-PAGE electrophoresis was performed using 4.5% stacking and 10% resolving gels [14] with a Mini-PROTEAN II electrophoresis cell (Bio-Rad) in 25 mM Tris, 190 mM glycine, and 2 mM SDS buffer. Proteins were transferred onto Trans-Blot nitrocellulose membrane (Bio-Rad) and PGHS-2 was immunodetected as we described previously [2].

Statistical Analysis

Data are expressed as the means ± SEM and experiments were performed on tissue pairs (control ear and ear administered with proinflammatory agent), with the determinations fit simultaneously to pairs of ears in order to obtain parameter estimates free of animal-to-animal variation. Statistical significance was assessed by the one-tailed Student's *t*-test for unpaired samples, with $P < 0.05$ regarded as significant.

RESULTS AND DISCUSSION

The accumulation of neutrophils and monocytes/macrophages is a characteristic feature of dermal inflammatory responses. Topical TPA application resulted in a gradual increase in MPO levels, which was taken as an index of polymorphonuclear cell infiltration. Influx of monocyte/macrophages typically occurs later than neutrophil influx, and the increase in tissue NAG levels can be used as an indicator of the presence of mononuclear cells [11]. This leukocyte extravasation into inflamed areas involves a complex interaction of leukocytes with the endothelium through regulated expression of surface adhesion molecules. Among them, L-selectin appears to play a key role in the initial attachment of circulating leukocytes to the endothelium [15]. Our previous results show that sialidase, which is widely used to remove carbohydrate moieties, which are the counter-receptors for selectins [16], significantly reduced leukocyte adhesion to endothelium stimulated by *N*-formyl-Met-Leu-Phe [17]. This effect of sialidase was observed a few minutes after intravenous administration of 0.01–0.1 mU per mouse.

Our data show that phorbol ester increased NAG levels nearly 2-fold at 6 hr, when MPO levels and ear edema were also significantly raised (Table 1). This leukocyte migration was accompanied by the enhancement of eicosanoid levels (Table 2) and PGHS-2 induction (Fig. 1). Sialidase administration (0.01–0.1 mU per mouse, i.v.) before TPA appli-

TABLE 1. Effect of sialidase treatment on cellular infiltration and edema formation

Treatments	MPO levels	NAG levels	Ear edema
Control	0.02 ± 0.01	19.1 ± 0.5	
TPA	81.3 ± 2.7	41.2 ± 1.9	123 ± 4
TPA + sialidase (0.01)	17.1 ± 0.4*	24.1 ± 1.1*	83 ± 4*
TPA + sialidase (0.1)	11.3 ± 0.3*	21.2 ± 1.4*	69 ± 3*
AA	2.3 ± 0.2	18.3 ± 0.4	126 ± 6
AA + sialidase (0.01)	0.7 ± 0.1*	ND	127 ± 5
AA + sialidase (0.1)	0.4 ± 0.1*	ND	131 ± 3

Cellular infiltration (MPO and NAG levels are expressed as mU/ear) and ear edema (expressed as % weight increase) were tested 6 or 1 hr after edema induction by TPA (6 μg) or AA (0.5 mg), respectively. Sialidase (0.01–0.1 mU per mouse) was administered intravenously 15 min before phlogogen application. Results are means ± SEM of 3–4 mice.

* Significantly different ($P < 0.05$ or less) from non-treated group; ND, not determined.

TABLE 2. Effect of sialidase treatment on eicosanoid levels in TPA- or AA-induced ear edema

Treatments	PGE_2 (ng/ear)	LTB_4 (pg/ear)	6-keto-$PGF_{1\alpha}$ (pg/ear)
Control	9 ± 1	5 ± 1	64 ± 9
TPA (6 μg)	ND	1133 ± 53*	512 ± 11*
TPA + sialidase	ND	275 ± 2†	203 ± 7†
AA (0.5 mg)	56 ± 2*	28 ± 2	ND
AA + sialidase	48 ± 3	31 ± 3	ND

Eicosanoid tissue levels were tested in TPA- and AA-induced ear edema, 6 and 1 hr, respectively, after phlogogens were applied. Sialidase (0.1 mU per mouse) was administered intravenously 15 min before phlogogen application. Values are means ± SEM of 4–5 animals.

* $P < 0.05$ vs control samples, † $P < 0.05$ vs non-treated samples. ND, not determined.

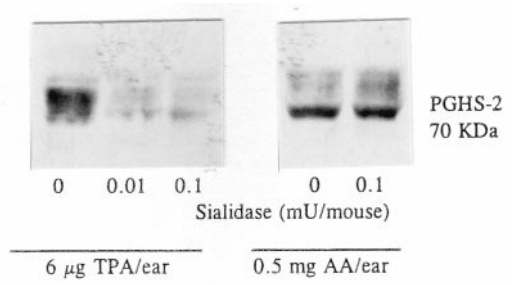

FIG. 1. The impairment of cell influx by sialidase markedly reduced TPA-induced PGHS-2 expression. Mice received sialidase (0.01–0.1 mU per mouse, intravenously) 15 min before TPA or AA application. Edema was measured 6 or 1 hr after TPA- or AA-induced ear edema, respectively. Tissue was homogenized and PGHS-2 protein levels measured. The Western blot is representative of 4 experiments.

cation markedly reduced cell influx, edema formation (Table 1), and eicosanoid levels in inflamed tissue (Table 2). Interestingly, sialidase treatment also significantly decreased PGHS-2 overexpression induced by TPA (Fig. 1). Thus, these results suggest that skin leukocyte accumulation induced by TPA application is necessary for the progression of the inflammatory reaction as well as for the overexpression of PGHS-2. In contrast, topical application of AA resulted in a marked enhancement of eicosanoid levels, whereas we observed a slight time-dependent increase in mouse ear MPO levels. Moreover, sialidase treatment failed to prevent edema, eicosanoid formation (Tables 1 and 2), and PGHS-2 overexpression induced by AA (Fig. 1). These data indicate that AA and/or AA metabolites may be involved in the induction of PGHS-2 expression in this experimental model of inflammation. Furthermore, we can hypothesize that skin PGHS-2 overexpression induced by TPA application could be induced by AA and/or AA metabolite release by leukocyte infiltrated during the inflammatory process.

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