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Are the anti-inflammatory effects of dexamethasone responsible for inhibition of the induction of enzymes involved in prostanoid formation in rat carrageenin-induced pleurisy?

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Abstract

Since anti-inflammatory steroids modulate multiple gene expression, including the expression of prostaglandin H synthase-2 and phospholipase A_2, at the molecular level, we studied the effects of dexamethasone on rat carrageenin-induced pleurisy to elucidate whether regulation of phospholipase A_2 and prostaglandin H synthase-2 expression is the primary mechanism of its anti-inflammatory action. Suppression of plasma exudation by a lower dose of dexamethasone (0.3 mg/kg) was almost equal to that by aspirin (100 mg/kg), but that by higher dexamethasone doses (3 and 30 mg/kg) was considerably stronger, suggesting the involvement of effects other than that on prostanoid formation. The lower dose of dexamethasone also significantly reduced the pleural exudate neutrophil count and prostanoid levels. However, this dose affected neither the prostaglandin H synthase-2 level nor the phospholipase A_2 activity in the exudate cells. The prostaglandin H synthase-2 level was affected only at the higher doses, while phospholipase A_2 activity was not. These results suggest that the anti-inflammatory effects of dexamethasone in acute inflammation cannot be ascribed to direct interference with prostanoid formation via suppression of phospholipase A_2 and prostaglandin H synthase-2 expression.

Keywords: Carrageenin-induced pleurisy, rat; Anti-inflammatory steroid; Dexamethasone; Prostaglandin H synthase-2 (cyclooxygenase-2); Phospholipase A_2

1. Introduction

Glucocorticoids are potent anti-inflammatory agents widely used in various diseases, such as rheumatoid arthritis, systemic lupus erythematosus, asthma and other chronic inflammatory and autoimmune diseases. Information concerning their effects at the molecular level is now accumulating (Barnes and Adcock, 1993; DiDonato et al., 1996; Cato and Wade, 1996; Goppelt-Struebe, 1997). Glucocorticoids exert their effects by binding to a cytoplasmic glucocorticoid receptor within the target cells. The glucocorticoid–receptor complex then translocates to the nucleus, where it binds to cis-acting DNA sequences known as glucocorticoid response elements, and modulates transcription of specific target genes in a positive or negative manner. Furthermore, it has recently been found that the glucocorticoid–receptor complex can modulate transcription, not through direct binding to glucocorticoid response elements but through interference with the activity of other transcription factors, such as nuclear factor-κB (Siebenlist et al., 1994) and activator protein-1 (Angel and Karin, 1991). Thus, glucocorticoids exert their effects at the molecular level by interfering with multiple signaling pathways. Consequently, a wide variety of proinflammatory and immunoregulatory genes are negatively regulated by glucocorticoids, including those coding for interleukin-1β, tumor necrosis factor-α, interleukin-2, interleukin-6, interleukin-8, granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-2 receptor, intercellular adhe-

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sion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), reduced upon activation normal T expressed and secreted (RANTES), E-selectin, collagenases and an inducible type of nitric oxide synthase. Phospholipase A_2 (Murakami et al., 1997) and prostaglandin H synthase-2 (Herschman, 1996), both of which play a key role in prostanoid formation, should also be included in this list (Goppelt-Struebe, 1997).

In the acute inflammatory model, inflammatory reactions are characterized by leukocyte infiltration and plasma exudation. Dexamethasone, a clinically widely used glucocorticoid, suppresses accumulation of leukocytes at the site of inflammation (Ishikawa et al., 1969). The steroid also suppresses plasma exudation (Church and Miller, 1978; Tsurufuji et al., 1980) and decreases the level of prostanoid (Salmon et al., 1983) in acute inflammatory models. Glucocorticoids may exert these anti-inflammatory effects by regulating the expression of genes coding molecules involved in inflammation. However, whether or not glucocorticoids affect the expression of these genes equally is obscure.

In rat carrageenin-induced pleurisy, a high level of prostaglandin H synthase-2 is detectable from 3 to 7 h in neutrophils and mononuclear leukocytes (Harada et al., 1994, 1996), and from 9 to 24 h in pleural mesothelial cells (Hatanaka et al., 1999). In the early stage, selective prostaglandin H synthase-2 inhibitors suppress plasma exudation and preferentially reduce the prostaglandin E_2 level, but not the levels of 6-keto-prostaglandin $F_{1\alpha}$ or thromboxane B2 in the pleural exudate. These results suggest that prostaglandin E_2, generated via prostaglandin H synthase-2 by leukocytes in the exudate, may play an important role in plasma exudation in the early stage.

The aim of the present study was to elucidate whether or not regulation of phospholipase A_2 and prostaglandin H synthase-2 expression is the primary mechanism of the anti-inflammatory effects of glucocorticoids in acute inflammation. For this purpose, we evaluated the effects of dexamethasone on plasma exudation, leukocyte accumulation, prostanoid levels, prostaglandin H synthase isoform levels and phospholipase A_2 activities in rat carrageenin-induced pleurisy.

2. Materials and methods

2.1. Carrageenin pleurisy

All experiments were performed according to the Guideline for Animal Experimentation of Kitasato University. Carrageenin pleurisy was induced in male Sprague–Dawley rats (9–10 weeks old, specific pathogen free), purchased from SLC (Hamamatsu, Japan), by intrapleural injection of 0.2 ml of 2% λ-carrageenin (Zushi Chemical, Zushi, Japan) under light ether anesthesia according to the method described previously (Harada et al., 1996). Dexamethasone (0.3, 3 and 30 mg/kg, Banyu Pharmaceutical, Tokyo, Japan) had been intraperitoneally injected 2 h before; and aspirin (100 mg/kg, Sanko Seiyaku Kogyo, Tokyo, Japan) suspended with 1% carboxymethyl cellulose in saline had been administered orally 1 h before the injection of carrageenin. The rats were exsanguinated under ether anesthesia 5 h after the injection of carrageenin, and pleural exudate was harvested. For the control, the pleural cavity of normal control rats was washed with 2 ml of 0.9% NaCl solution and the lavage was then harvested. Cells in the harvested lavage and exudate were counted using an improved Neubauer cell count plate after fixation with Turk's solution.

Cells in the harvested fluid were washed twice with 10 mM phosphate-buffered saline (pH 7.2) containing 1 mM EDTA by centrifugation at $200 \times g$ for 5 min at 4°C, and were stored at −80°C prior to Western blot analysis. In separate experiments, the harvested fluid was centrifuged at $200 \times g$ for 5 min to measure the phospholipase A_2 activities of the supernatant and of the cell pellet, separately. The pellet was washed twice with 10 mM phosphate-buffered saline (pH 7.2). Both fractions were stored at −80°C until phospholipase A_2 activity was measured.

For the prostanoid assay, 2 ml of saline solution containing 20 μM indomethacin and 15.4 mM EDTA were injected into the pleural cavity immediately after exsanguination. The pleural fluid was then collected and immediately frozen at −80°C until clean-up. In separate experiments, rats were intravenously injected with pontamine sky blue (50 mg/kg, Tokyo Kasei, Tokyo, Japan) 20 min before exsanguination for assessment of the plasma exudation rate. The amount of dye in the pleural fluid was measured spectrophotometrically by absorption at 630 nm and normalized according to the concentration of the dye in the serum. The detection limit for the dye was approximately 2.5 μg/sample.

2.2. Prostanoid assay

The prostanoid level was determined as described previously (Harada et al., 1996). Briefly, the frozen pleural fluid was thawed and centrifuged at $2000 \times g$ for 10 min. The supernatant was acidified to pH 3 with 1 N HCl and then again centrifuged at $2000 \times g$ for 10 min. The resulting supernatant was applied to a Sep-Pak C18 column (Waters Associates, Milford, MA, USA). After the separation of prostaglandin E_2, thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ by high performance liquid chromatography (HPLC), prostanoid was measured by enzyme-immunoassay kits (Cayman Chemical, Ann Arbor, MI, USA). The overall recovery amounts assessed by addition of authentic prostanoid to the sample were $57.8 \pm 1.9\%$ ($n = 13$), $44.3 \pm 1.3\%$ ($n = 13$) and $61.5 \pm 1.6\%$ ($n = 13$) for prostaglandin E_2, thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$, respectively. The detection limit for each prostanoid was approximately 0.1 to 0.06 ng/sample.

2.3. Western blot analysis

Western blot analysis for prostaglandin H synthase-1 and prostaglandin H synthase-2 of the exudate cells was performed by the method described previously (Harada et al., 1994). In brief, the frozen exudate cell pellet was thawed and suspended in 20 mM Tris–HCl buffer (pH7.4) containing 5 mM tryptophan and 2 mM phenylmethyl sulfonyl fluoride (Wako Pure Chemicals, Osaka, Japan), and then sonicated for 1 min at 4°C. The homogenate was then solubilized in 0.5% Tween 20 and centrifuged at $140000 \times g$ for 1 h at 4°C. The resulting supernatant was diluted with an equal volume of the sampling buffer of the following composition: 0.1 M Tris–HCl (pH 6.8), 20% glycerol, 0.1 mg/ml methyl green and 2% sodium dodecyl sulfate (SDS). The solubilized protein (40 to 50 μg protein/lane) was subjected to SDS-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking with Block Ace (Dainippon Pharmaceutical, Osaka, Japan), the blot membrane was incubated with rabbit anti-bovine prostaglandin H synthase-1 antiserum (Ishimura et al., 1993) or rabbit anti-murine prostaglandin H synthase-2 antiserum (Cayman Chemical, Ann Arbor, MI, USA). After incubation with goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Organon Teknika-Cappel products, Durham, NC, USA), the membrane was stained with Konica immunostain HRP-1000 (Konica, Tokyo, Japan).

2.4. Measurement of phospholipase A_2 activities

The frozen cell pellet was thawed and suspended in 25 mM HEPES buffer (pH 7.2) containing 2 mM EDTA, 1 mM EGTA, 100 μM leupeptin and 50 μM pepstatin A, to measure the secretory phospholipase A_2 activities, or in 50 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 100 μM leupeptin, 50 μM pepstatin A, 100 mM sodium fluoride, 200 μM sodium orthovanadate, 1 μM microcystin LR, 10 μM genistein and 1 μM staurosporine to measure the cytosolic phospholipase A_2 activities (1×10^8 cells/ml). It was then sonicated for 3 s at 4°C by an ultrasonic disrupter (XL2005 Microson™ Cell Disrupter, Heat Systems, Farmingdale, NY, USA).

The activities of non-pancreatic secretory phospholipase A_2 in the cell homogenate and the supernatant of the pleural lavage and exudate were measured by the method of Tojo et al. (1993). Samples (20–135 μg protein) were incubated for 30 min at 40°C with a mixed micelle (final volume 100 μl) consisting of 1 mM 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (Avanti polar Lipids, Alabaster, AL, USA) and 2 mM sodium deoxycholate in 0.1 M Tris–HCl buffer (pH 8.0) containing 150 mM NaCl and 10 mM $CaCl_2$ and 1 mg/ml bovine serum albumin. The reaction was stopped by addition of 400 μl of Dole's reagent. Hydrolyzed oleic acid was extracted into the upper heptane layer and the extracted fatty acid was derivatized and analyzed by HPLC. Specific secretory phospholipase A_2 activity was calculated by subtraction of non-enzymatic hydrolysis of the substrate (less than 1% of the total phospholipid added).

The activity of cytosolic phospholipase A_2 was determined by the method of Kramer et al. (1991). Twenty microliters of cell homogenate (2–6 μg protein) was incubated for 15 min at 37°C in a mixed micelle (final volume 200 μl) consisting of 1-palmitoyl-2-[^{14}C]arachidonyl-phosphatidylcholine (2.5 μM, 667 Bq/nmol, NEN, Boston, MA, USA and Avanti Polar Lipids, Alabaster, AL, USA) and 1.25 μM of 1,2-dioleoylglycerol (Avanti Polar Lipids, Alabaster, AL, USA) in 100 mM glycine–NaOH buffer (pH 9.5) containing 150 mM NaCl, 5 mM $CaCl_2$ and 2 mM dithiothreitol. The reaction was stopped by the addition of 1.25 ml of Dole's reagent. Free [^{14}C]arachidonic acid was extracted in *n*-heptane, and the radioactivity was counted by a liquid-scintillation counter (TRI-CARB Liquid Scintillation Analyzer Model 2200CA, Packard Instrument, Meriden, CT, USA). Specific cytosolic phospholipase A_2 activity was calculated by subtraction of non-enzymatic hydrolysis of the substrate (less than 0.5% of the total radioactivity added).

Protein was measured by BCA protein Assay Reagent (Pierce, Rockford IL, USA).

2.5. Data analysis

Results were expressed as the mean ± S.E.M. from n experiments. Fischer's or Scheffe's test was used to evaluate significant differences between means. A *P*-value of less than 0.05 was considered statistically significant and is indicated by an asterisk in the figures.

2.6. Materials

Arachidonyl trifluoromethyl ketone (AACOCF3), a cytosolic phospholipase A_2 inhibitor (Street et al., 1993), was purchased from Cayman Chemical. Antibody for secretory phospholipase A_2 was raised against phospholipase A_2 released from thrombin-stimulated rat platelets (Nakano et al., 1990).

3. Results

3.1. Anti-inflammatory effects

The amount of pleural fluid collected from normal control rats was 0.06 ± 0.01 ml ($n = 10$), and that of pontamine sky blue that leaked in the pleural cavity after intravenous injection over 20 min was 3.48 ± 0.67 μg ($n = 7$). These parameters were not significantly affected by pretreatment with aspirin or dexamethasone (data not

shown). Intrapleural injection of carrageenin caused the accumulation of a volume of pleural exudate that reached 1.76 ± 0.13 ml ($n = 8$); and the plasma exudation rate, estimated from the exuded dye amount, reached 202 ± 19 μg ($n = 8$) 5 h after the injection. Dexamethasone dose-dependently suppressed both the accumulation of pleural exudate and the plasma exudation rate, as did aspirin (Fig. 1). It is noteworthy that higher doses of dexamethasone (3 and 30 mg/kg) inhibited both parameters more effectively than did aspirin. The total count of leukocytes harvested from the lavage fluid from the pleural cavity of normal control rats was $13.0 \pm 0.5 \times 10^6$ cells ($n = 9$). Neutrophils and mononuclear leukocytes accounted for $2.6 \pm 0.6\%$ and $77.1 \pm 1.3\%$, respectively. The remaining cells included eosinophils ($12.5 \pm 1.5\%$) and mast cells ($7.4 \pm 1.1\%$). The total leukocyte count transiently decreased to $7.3 \pm 0.6 \times 10^6$ ($n = 3$) 1 h after intrapleural injection of carrageenin (neutrophils, $0.2 \pm 0.04 \times 10^6$; mononuclear leukocytes, $4.9 \pm 0.6 \times 10^6$). The counts of neutrophils and mononuclear leukocytes recovered to the lavage fluid level by 3 and 5 h, respectively, which indicated the start of intrapleural infiltration of leukocytes. The total leukocyte count was $159 \pm 18 \times 10^6$ ($n = 5$) with $92.5 \pm 0.6\%$ of neutrophils and $5.8 \pm 0.5\%$ of mononuclear leukocytes

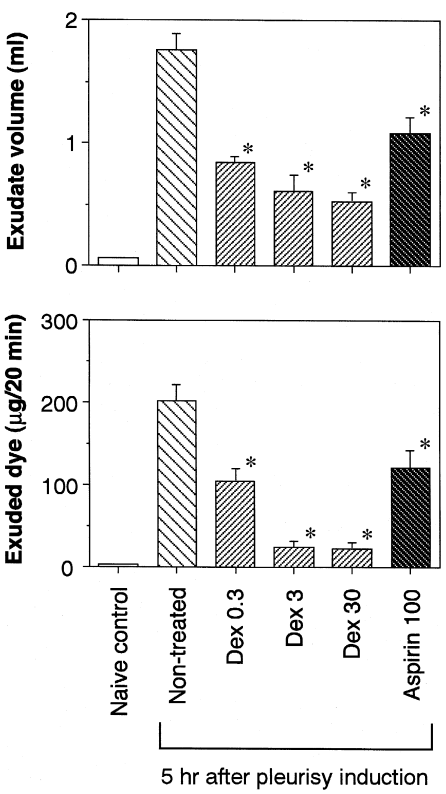

Fig. 1. Effects of dexamethasone (Dex; 0.3, 3, 30 mg/kg) and aspirin (100 mg/kg) on pleural exudate volume (upper panel) and the amount of dye exuded over 20 min (lower panel) at 5 h after carrageenin injection. Naive control refers to the pleural fluid collected from the pleural cavity of normal control rats injected with the dye 20 min before exsanguination. Each value indicates the mean ± S.E.M. of 4 to 10 rats.

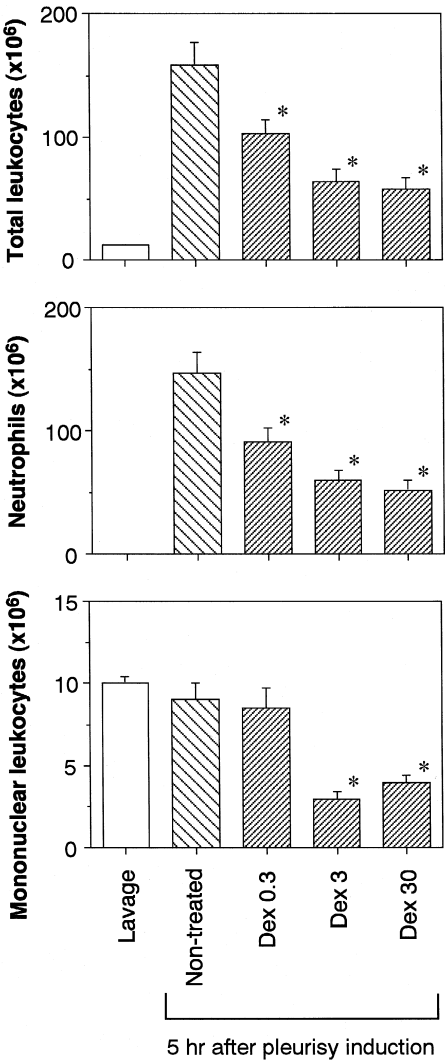

Fig. 2. Effects of dexamethasone on numbers of leukocytes in the pleural exudate at 5 h after carrageenin injection. Lavage refers to the pleural fluid leukocytes collected by lavage from the pleural cavity of normal control rats. Each value indicates the mean ± S.E.M. of three to nine rats.

5 h after pleurisy induction. Pretreatment with dexamethasone dose-dependently decreased the total number of leukocytes in the exudate (Fig. 2). The lowest dose of dexamethasone (0.3 mg/kg) significantly reduced the number of neutrophils to 62% of the non-treated control, but did not affect that of mononuclear leukocytes. In contrast, higher doses of dexamethasone (3 and 30 mg/kg) significantly reduced accumulation of both types of leukocytes; particularly the mononuclear leukocyte count was reduced below the starting point at 1 h.

3.2. Effects on levels of prostanoid and prostaglandin H synthase isoforms

The levels of prostaglandin E_2, thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ in the lavage fluid from normal control rats were 0.14 ± 0.04, 0.32 ± 0.10 and 0.40 ± 0.17

ng/rat ($n = 6$), respectively, and these levels were significantly increased to 1.12 ± 0.14, 3.03 ± 0.32 and 3.45 ± 0.49 ng/rat ($n = 11$, 16 and 8), respectively, 5 h after pleurisy induction. Dexamethasone and aspirin significantly suppressed all of the prostanoids measured (Fig. 3). In pleural lavage cells harvested from normal control rats, only prostaglandin H synthase-1, and not prostaglandin H synthase-2, was detectable (data not shown). In the exudate cells 5 h after the induction of pleurisy, both prostaglandin H synthase-1 and prostaglandin H synthase-2 were detectable at high levels (Fig. 4). The lowest dose of dexamethasone (0.3 mg/kg) did not affect the prostaglandin H synthase-2 level, but the higher doses reduced it to below the detection limit. However, the steroid did not affect the prostaglandin H synthase-1 level at any dose tested.

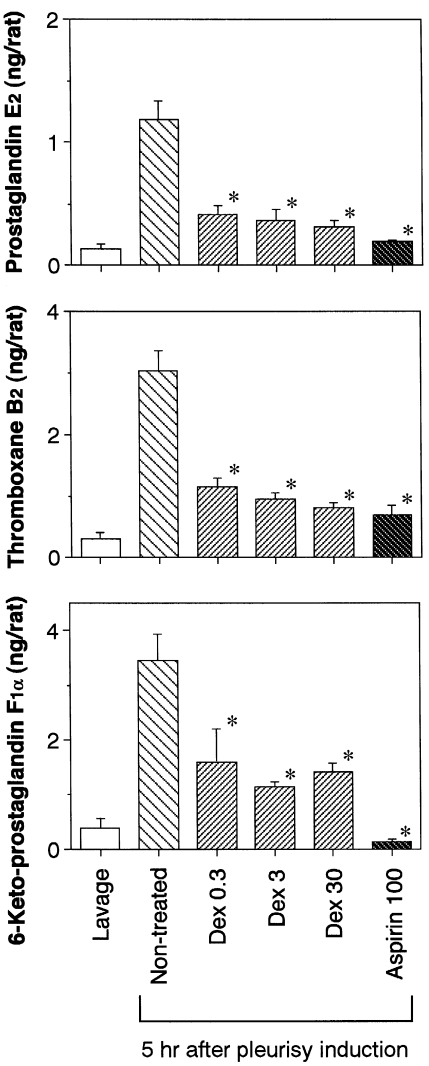

Fig. 3. Effects of dexamethasone and aspirin on levels of prostanoids at 5 h after carrageenin injection. Lavage refers to the fluid washed from the pleural cavity of normal control rats. Each value indicates the mean ± S.E.M. of 4 to 16 rats.

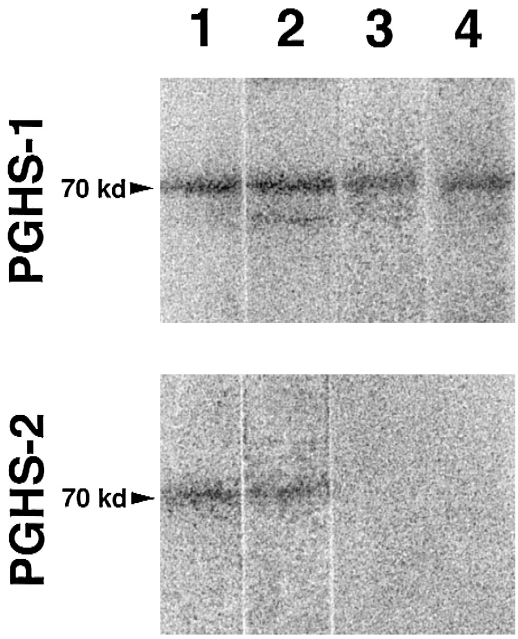

Fig. 4. Western blot analysis showing the effect of dexamethasone on the levels of prostaglandin H synthase-1 (PGHS-1) and prostaglandin H synthase-2 (PGHS-2) in pleural exudate cells at 5 h after carrageenin injection. Upper panel, prostaglandin H synthase-1; lower panel, prostaglandin H synthase-2. Lane 1, Non-treated control; Lane 2, dexamethasone treated (0.3 mg/kg); Lane 3, dexamethasone treated (3 mg/kg); Lane 4, dexamethasone treated (30 mg/kg). The position of the 70-kDa molecular marker protein is indicated. Similar results were obtained from two additional sets of experiments.

3.3. Effect on phospholipase A_2 activities

Oleic acid obtained by hydrolysis from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol was detected in the supernatant of the pleural lavage fluid of normal control rats and also in the supernatant of the pleural exudate 5 h after pleurisy induction, the respective specific activities being 3.00 ± 0.46 nmol/min/mg protein ($n = 6$) and 3.51 ± 0.22 nmol/min/mg protein ($n = 5$) (Fig. 5). The latter activity was inhibited to 10% of the original level by addition of anti-rat platelet secretory phospholipase A_2 antibody (220 μg/ml), but not by AACOCF3 (data not shown). This activity was also detected in the cell homogenates of pleural lavage fluid and exudate, the specific activities being 6.07 ± 1.93 nmol/min/mg protein ($n = 5$) and 5.95 ± 0.33 nmol/min/mg protein ($n = 8$), respectively (Fig. 6). The latter activity was also significantly inhibited by the antibody against secretory phospholipase A_2 to 22.9% of the original level. Therefore, the oleic acid releasing activity appeared to be the actions of secretory phospholipase A_2. The specific activities of secretory phospholipase A_2 in the lavage fluid and the exudate were almost equal in both the supernatant (Fig. 5) and the cell homogenate (Fig. 6), suggesting that no induction of secretory phospholipase A_2 had taken place. Pretreatment of

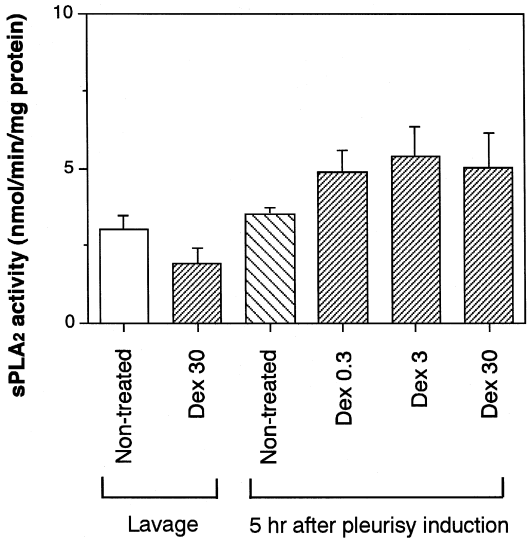

Fig. 5. Effects of dexamethasone on secretory phospholipase A_2 activity in the supernatant of lavage fluid and pleural exudate at 5 h after carrageenin injection. Each value indicates the mean ± S.E.M. of four to six rats.

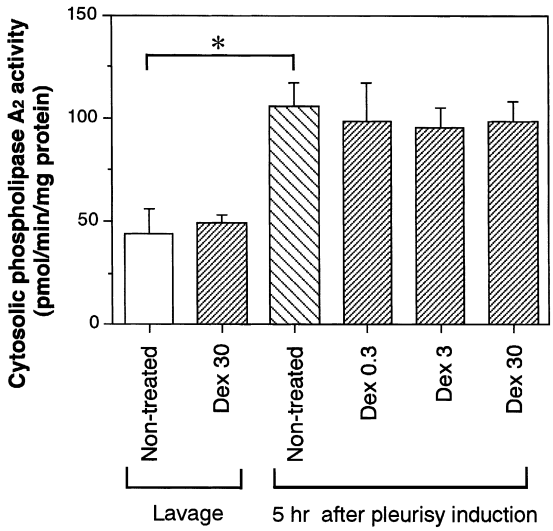

Fig. 7. Effects of dexamethasone on cytosolic phospholipase A_2 activity in the cell homogenate of lavage fluid and pleural exudate at 5 h after carrageenin injection. Each value indicates the mean ± S.E.M. of four to seven rats.

rats with dexamethasone (0.3, 3 and 30 mg/kg) never significantly affected secretory phospholipase A_2 activity in either supernatant or cell homogenate.

The cell homogenates of the pleural lavage fluid and the exudate also hydrolyzed 1-palmitoyl-2-[^{14}C] arachidonyl-phosphatidylcholine to release [^{14}C] arachidonic acid with a specific activity of 43.9 ± 11.9 pmol/min/mg protein ($n = 5$) and 106 ± 11.3 pmol/min/mg protein ($n = 7$) (Fig. 7). The latter activity was inhibited by AACOCF3 with an IC50 value of 90 ± 9 nM ($n = 3$), but not by the antibody against secretory phospholipase A_2 (data not shown), and appeared to be cytosolic phospholipase A_2 activity. Pretreatment of rats with dexamethasone also never significantly affected the cytosolic phospholipase A_2 activity either in the lavage cells or in the exudate cells.

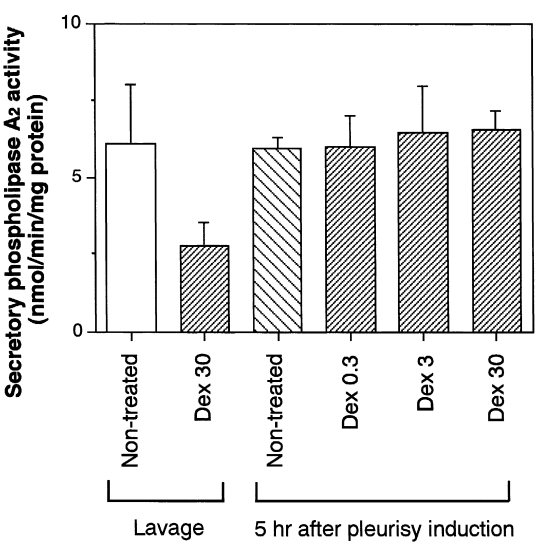

Fig. 6. Effects of dexamethasone on secretory phospholipase A_2 activity in the cell homogenate of lavage fluid and pleural exudate at 5 h after carrageenin injection. Each value indicates the mean ± S.E.M. of four to eight rats.

4. Discussion

4.1. Effects on phospholipase A_2 activities

Prostanoid synthesis is initiated when stimuli activate one or more types of phospholipase A_2, causing the release of precursor arachidonic acid from the membrane phospholipids. The released arachidonic acid is then converted to prostaglandin H_2 by the action of prostaglandin H synthase. Depending on the nature of the additional enzymes present in the stimulated cells, prostaglandin H_2 can then be converted to various prostanoids, including prostaglandin E_2, prostaglandin $F_{2\alpha}$, prostaglandin I_2, and thromboxane A_2.

There are at present three types of mammalian phospholipase A_2 whose primary structure has been deduced from the gene structure: pancreatic secretory phospholipase A_2, non-pancreatic secretory phospholipase A_2 and cytosolic phospholipase A_2 (Murakami et al., 1997). Secretory phospholipase A_2 and cytosolic phospholipase A_2 are induced by proinflammatory stimuli, including interleukin-1, interleukin-6, tumor necrosis factor-α and lipopolysaccharide in cultured cells, and the induction is suppressed by dexamethasone (Murakami et al., 1997). Different reports specify the involvement of different types of phospholipase A_2 in the provision of arachidonic acid for prostanoid formation in vitro. Consequently, it is still a

matter of controversy which type of phospholipase A_2 is active in the provision of arachidonic acid for the generation of prostanoids in vivo as well as in vitro (Murakami et al., 1997).

Because of the leukocyte infiltration and the accumulation of pleural exudate, the total activity of secretory phospholipase A_2 in the pleural cavity 5 h after carrageenin injection increased by about 100 times that in the lavage fluid of normal control rats. However, the specific activities of secretory phospholipase A_2 in the two groups of animals did not differ significantly (Figs. 5 and 6), suggesting that no induction of this enzyme had occurred. In any event, dexamethasone did not affect the secretory phospholipase A_2 activity in either the supernatant of the pleural exudate or in the cell homogenate (Figs. 5 and 6). Moreover, the secretory phospholipase A_2 inhibitor thielocin $A_{1\beta}$ does not reduce prostanoid levels even at an anti-inflammatory dose in rat carrageenin-induced pleurisy (Tanaka et al., 1993). Therefore, secretory phospholipase A_2 activity may not be involved in the release of arachidonic acid for prostanoid formation in this model. In contrast with the specific activity of secretory phospholipase A_2, that of cytosolic phospholipase A_2 increased after the induction of pleurisy (Fig. 7), suggesting the induction of cytosolic phospholipase A_2 or enhancement of the activity. However, cytosolic phospholipase A_2 activity was not affected even by the higher doses of dexamethasone (Fig. 7). Since we measured phospholipase A_2 activity in the presence of a large amount of substrate, any effect of lipocortins, which are induced by glucocorticoids (Flower and Rothwell, 1994) and interfere with phospholipase A_2 activities by binding to substrate phospholipids (Davidson et al., 1987), may be minimized. However, the possibility that lipocortin-like protein(s) induced by dexamethasone interfere with phospholipase A_2 in the inflammatory site cannot be excluded. At all events, the present results suggest that reduction of the prostanoid levels in pleural exudate by dexamethasone cannot be ascribed to suppression of phospholipase A_2 enzyme induction.

4.2. Effect on the prostaglandin H synthase-2 level

Two isoforms of prostaglandin H synthase have been identified. Prostaglandin H synthase-1 is expressed constitutively in most types of cells. Prostaglandin H synthase-2 gene expression is induced by a wide variety of biological stimuli including pro-inflammatory cytokines and growth factors such as interleukin-1β and tumor necrosis factor-α, and by a number of pharmacological stimuli such as phorbol esters and calcium ionophore in appropriate cells (Herschman, 1996). Such prostaglandin H synthase-2 gene expression is regulated by nuclear factor-κB (Yamamoto et al., 1995) and is suppressed by dexamethasone (Herschman, 1996). In the early stage of the rat carrageenin-induced pleurisy model, prostaglandin H synthase-2 is expressed in mononuclear leukocytes and neutrophils (Harada et al., 1994), and interleukin-1β and tumor necrosis factor-α activities are detectable in the pleural exudate (Utsunomiya et al., 1994). The expression of these pro-inflammatory cytokines is also down-regulated by dexamethasone (Barnes and Adcock, 1993), suggesting the presence of indirect down-regulation of the prostaglandin H synthase-2 expression by dexamethasone. Prostaglandin H synthase-2 expression was affected only by the higher doses of dexamethasone (3 mg/kg) (Fig. 4), and so decreased levels of prostaglandin H synthase-2 and prostanoids were not correlated. We found previously that selective prostaglandin H synthase-2 inhibitors suppressed plasma exudation and preferentially reduced the level of prostaglandin E_2 in the inflammatory site, suggesting that prostaglandin E_2 may be generated via prostaglandin H synthase-2 expressed in neutrophils and mononuclear leukocytes (Harada et al., 1996). Thus, the significant decreases in prostanoid levels, particularly prostaglandin E_2 levels, caused by the lowest dose of dexamethasone could not be attributed to suppression of prostaglandin H synthase-2 expression. All of these results indicate that the dose of dexamethasone required to affect prostaglandin H synthase-2 expression is higher than that needed to suppress prostanoid formation in the inflammatory site.

4.3. Effects on leukocyte infiltration

Leukocyte infiltration is initiated by the activation of the leukocytes by chemotactic factors, followed by rolling on the venular endothelium, adhesion to the endothelium, passage through the endothelial layer, and migration into the interstitial spaces after passage through the basement membrane (Oda et al., 1992). Dexamethasone appears to have a number of targets for inhibiting leukocyte infiltration. It inhibits gene expression of cell adhesion molecules (Cronstein et al., 1992) and of chemotactic pro-inflammatory cytokines (Barnes and Adcock, 1993; DiDonato et al., 1996; Cato and Wade, 1996).

Neutrophils are activated by many chemotactic factors, including formyl-Met–Leu–Phe (fMLP), complement component C5a des Arg, leukotriene B_4 and platelet activating factor. Pro-inflammatory cytokines, such as interleukin-1β, which is generated by leukocytes (Dinarello, 1984; Cassatella, 1995) and is detected in this model (Utsunomiya et al., 1994), also induce neutrophil activation (Cybulsky et al., 1986; Utsunomiya et al., 1996). The glucocorticoids also inhibit the expression of the collagenase gene family interfering with activator protein-1 (Yang-Yen et al., 1990). Observations using intravital microscopy in the hamster cheek pouch demonstrate that lower doses of dexamethasone (0.3 mg/kg) and a collagenase inhibitor suppress the passage through the basement membrane of neutrophils which are activated by leukotriene B_4 or fMLP (Oda and Katori, 1992; Oda et al., 1995). Therefore, the process of inhibition of the gene expression of cell adhesion molecules, pro-inflammatory cytokines

and collagenases seems to be the principal target of dexamethasone for suppression of the neutrophil infiltration.

The transient decrease in the leukocyte count 1 h after pleurisy induction may have been due to the cytotoxic effects of carrageenin on leukocytes (Catanzaro et al., 1971). Apoptosis might be involved in the decrease of the mononuclear leukocyte count induced by higher doses of dexamethasone, because of enhanced apoptosis of macrophages (Kanematsu and Suketa, 1997).

4.4. Effects on the prostanoid level

As mentioned above, leukocytes in the pleural exudate express prostaglandin H synthase-2 which is considered to be responsible for the production of prostaglandin E_2 (Harada et al., 1994, 1996). The lowest dose of dexamethasone, by which the prostaglandin H synthase-2 level and phospholipase A_2 activities were not affected, suppressed accumulation of neutrophils but not that of mononuclear leukocytes. The higher doses of dexamethasone suppressed accumulation of both types of the leukocyte with lowering prostaglandin H synthase-2 level below the detection limit. Although, more than 90% of the leukocytes in the exudate were neutrophils that infiltrated 5 h after pleurisy induction, the expression level of prostaglandin H synthase-2 was approximately 10 times as high in mononuclear leukocytes as in neutrophils (Harada et al., 1996). Furthermore, prostaglandin E synthase is identified to be inducible and its expression is deduced to be regulated by dexamethasone (Jakobsson et al., 1999). However, the level of each prostanoid was almost equally lowered by the steroid, while selective prostaglandin H synthase-2 inhibitors preferentially reduce the prostaglandin E_2 level, but not the levels of 6-keto-prostaglandin F1a or thromboxane B_2. Moreover, the rate of reduction of the prostanoid levels was well correlated with that of leukocyte count (Figs. 2 and 3). Prostanoid released in the absence of prostaglandin H synthase-2 may be produced via prostaglandin H synthase-1, expression of which was not affected by the steroid (Fig. 4). All of these facts, therefore, suggest that decreased accumulation of leukocytes, which produce prostanoids in the inflammatory site, might be one of the targets of dexametha-sone for lowering the prostanoid level in the pleural exudate.

4.5. Effects on plasma exudation

Pretreatment with dexamethasone dose-dependently suppressed plasma exudation (Fig. 1) and also reduced the level of prostanoids (Fig. 3). Prostanoids themselves do not induce plasma exudation but potentiate that induced by bradykinin or other mediators (Williams and Morley, 1973). The reduction by aspirin of prostanoid levels in pleural exudate was equal to, or somewhat stronger than, that by dexamethasone (Fig. 3). In contrast, although plasma exudation was almost equally suppressed by aspirin and the lowest dose of dexamethasone, the higher doses of dexamethasone caused stronger suppression of plasma exudation (Fig. 1). Suppression of plasma exudation by prostaglandin H synthase inhibition is thought to take place through the elimination only of the potentiation produced by prostanoids (Williams and Morley, 1973). In fact, treatment with non-selective inhibitors of prostaglandin H synthase-1 and prostaglandin H synthase-2 or with prostaglandin H synthase-2 selective inhibitors caused no more than 50% suppression of the plasma exudation in the rat carrageenin pleurisy model in spite of a marked reduction of the prostaglandin E_2 level (Harada et al., 1998). A low dose of dexamethasone (0.5 mg/kg) inhibits edema caused by bradykinin in the paws of mice, as is required by gene expression (Tsurufuji et al., 1980). It is also suggested that constriction of local vessels does not play an essential role in the anti-exudative effect of dexamethasone (Sugio and Tsurufuji, 1981). These facts, taken together, suggest that modulation of the sensitivity of the vascular tissue to mediators may be a more susceptible target for the anti-exudative effect of dexamethasone rather than interference with the expression of phospholipase A_2 or prostaglandin H synthase-2.

In conclusion, neither prostaglandin H synthase-2 level nor phospholipase A_2 activities was correlated with prostanoid levels and with the plasma exudation in the inflammatory site.

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