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Lipocortin 1 Mediates the Suppressive Effects of Dexamethasone on ConA-induced Proliferative Response and Nitric Oxide Production in Rat Splenic Leukocytes

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Lipocortin 1 has been proposed as a putative mediator of anti-inflammatory actions of glucocorticoids. We investigated the role of lipocortin 1 in the effect of dexamethasone using rat splenic leukocytes. Concanavalin A(ConA; 1 μg/ml) increased the leukocyte proliferation and nitric oxide(NO) generation, which were measured as [^{3}H]-thymidine uptake by the cells and nitrite accumulation in the culture media, respectively. Dexamethasone suppressed ConA-induced cell proliferation, in a concentration-dependent manner with EC_{50} around 50nM. The addition of anti-lipocortin 1(Anti-LC1) reversed dexamethasone effects: 0.24, 1.2, 6 μg/ml of Anti-LC1 reversed dexamethasone(50 nM)-induced suppression of thymidine uptake by 9±3%, 16±3%, 36±5%, respectively; 0.24, 1.2, and 6 μg/ml of Anti-LC1 reversed dexamethasone-induced decrease of nitrite concentration by 49±16%, 61±20%, 77±19%, respectively. The present data indicate that lipocortin 1 mediates, at least in part, glucocorticoids-induced suppression of leukocyte proliferation and blockade of NO generation.

Key Words : Lipocortin 1, Dexamethasone, Thymidine uptake, Nitric oxide production

INTRODUCTION

Glucocorticoids, which have profound anti-inflammatory actions, are thought to produce biological effects by altering gene expression in their target cells. There are probably multiple mechanisms by which glucocorticoids exert their anti-inflammatory effects. However, much attention has focused on one putative mediator, lipocortin 1, which is a member of the annexin family of Ca^{2+}- and phospholipid-binding proteins(Flower & Rothwell, 1994).

There is bunch of evidence supporting that lipocortin 1 may represent the second messenger of the anti-inflammatory effects of glucocorticoids. Glucocorticoids induce the synthesis of lipocortin 1 in several cell types in vitro(Croxtall & Flower, 1992; Wu et al, 1995) and in vivo(Goulding et al, 1990; Peers et al, 1993) while surgical or pharmacological adrenalectomy results in decreases in lipocortin 1 mRNA and tissue protein content(Vishwanath et al, 1992; Peers et al, 1993). Furthermore, specific antibodies to lipocortin 1(Anti-LC1) reverse the effects of glucocorticoids on inflammation(Perretti et al, 1994), cell growth and differentiation(Croxtall & Flower, 1992), and eicosanoid release(Violette et al, 1990). However, not all actions of glucocorticoids are mediated by lipocortin 1. On the other hand, there are a number of additional factors controlling the expression of lipocortin 1 other than glucocorticoids.

The mitogen concanavalin A(ConA) enhances the production of nitric oxide(NO) from macrophages as well as stimulates the proliferation of lymphocytes (Lee, 1994). Glucocorticoids inhibit the proliferation of many cell types in culture including ConA- stimulated lymphocytes(Blauer et al, 1991). The hormones also inhibit the induction of NO synthase in macrophages(Di Rosa et al, 1990). In the present paper we examined the role of lipocortin 1 in dexamethasone effects on ConA-stimulated proliferation of rat splenic leukocytes and NO production.

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METHODS

Materials

Sprague-Dawley rats, from Clea Japan, were bred and maintained in specific pathogen-free environment at the Asan Institute for Life Sciences in Seoul, Korea.

All reagents for cell culture were obtained from Gibco(Grand Island, NY); dexamethasone, ConA and Ficoll Hypaque were from Sigma(St. Lousis, MO); [^{3}H]-thymidine was from Amersham(Buckinhamshire, England).

Polyclonal antibody against Anti-LC1 was raised in New Zealand rabbits by repeated injection of antigen. The rabbits received 3 subcutaneous injection of recommbinant human lipocortin 1(Huh et al, 1996) with an interval of 3 or 4 weeks. Then, serum was obtained and immunoglobulins in serum was separated by protein A-agarose affinity chromatography(Gersten & Marchalonis, 1978). Following the purification, the antibody was confirmed by immunodiffusion test and western blot using lipocortin 1 as an antigen.

Cell culture

Spleens were obtained aseptically from 8 week old male Sprague-Dawley rats. Single-cell suspensions of spleen cells were prepared by passing through a stainless steel screen by using syringe plunger, and were centrifuged over Ficoll-Hypaque to eliminate erythrocytes(Lee, 1994). Splenic leukocytes were resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 mM 2-mercaptoethanol, 5 mM L-glutamine, 10 mM HEPES, and penicillin-streptomycin. The cells were cultured at 1×10^5 cells/well in 0.2 ml in a 96-well microtiter culture plate.

Measurement of cell proliferation

Splenic cells were stimulated with T cell mitogen ConA(1 μg/ml) in the presence or absence of dexamethasone(0.1 nM- 10 μM). To measure proliferation, [^{3}H]-thymidine uptake was determined in quadriplicate after 72hr of culture, following an 18hr incubation with 1 μCi of [^{3}H]-thymidine(specific activity = 5 mCi/mmole). The ConA dose chosen represents 70% maximal response on the dose-response curves with the same culture system. Anti-LC1 was added at a final concentrations of 0.24, 1.2 and 6 μg/ml. Fresh antibody was added each day during the culture.

Measurement of nitrite

The accumulation of nitrite in the culture supernatant of splenic cells was measured in triplicate after 48hr of culture using the microplate assay described by Ding et al(1988). In brief, the supernatant was mixed with an equal volume of Griess reagent(1% sulphanilamide / 0.1% naphthylethylenediamine dihydrochloride / 2.5% H_3PO_4) and incubated at room temperature for 10 min. The absorbance was read at 540 nm using $NaNO_2$ as a standard. The concentration of nitrite in cell-free medium alone was determined in each experiment and subtracted from the value obtained with cells.

Statistical analysis

Values are presented as means±SE. The statistical significance of differences between means was assessed by Student's t-test for paired and unpaired data or by analysis of variance followed by Fisher's Protected Least Significant Difference test when more than two groups were compared. Differences were considered significant at $P<0.05$.

RESULTS

Effect of dexamethasone on leukocyte proliferation

The stimulation of splenic leukocyts with ConA(1 μg/ml) increased thymidine uptake from 1,670±487 to 177,473±22,737 dpm/10^5 cells(refer to Fig. 2A). The addition of dexamethasone(0.1nM to 10 μM) inhibited splenic cell proliferation in a concentration dependent manner(Fig. 1). Dexamethasone at a final concentration of 50nM decreased ConA-stimulated thymidine uptake to 57±7% and this concentration of the drug was used to examine the effect of Anti-LC1.

Effect of Anti-LC1 on dexamethasone-induced suppression of proliferation

Dexamethasone(50nM) decreased ConA-induced thymidine uptake from 177,473±22,737 to 96,411 ± 19,384dpm/10^5cells(Fig. 2A). The addition of Anti-LC1 to culture medium partially reversed the suppressive effect of dexamethasone on leukocyte proliferation: 0.24, 1.2, 6 μg/ml of Anti-LC1 reversed 9 ±3%, 16±3%, 36±5%, respectively, of the sup-

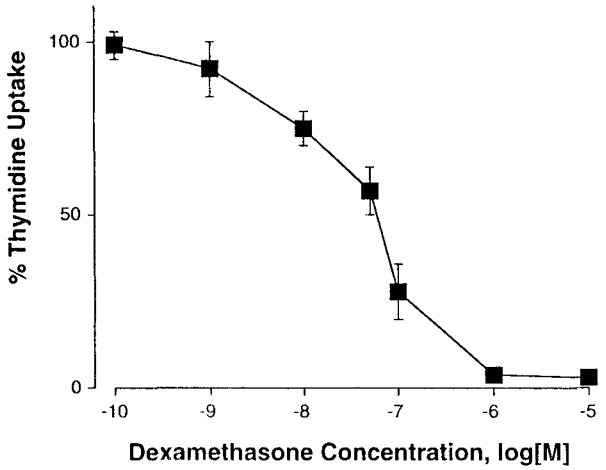

Fig. 1. The dose response curve of dexamethason on [^{3}H]-thymidine uptake by ConA-stimulated rat splenic leukocytes(n=5). Cells(1×10^5/well) were cultured with 1 μg/ml ConA in the absence or presence of 0.1nM - 10 μM dexamethasone. Ordinate represents % of ConA-stimulated thymidine uptake without dexamethasone.

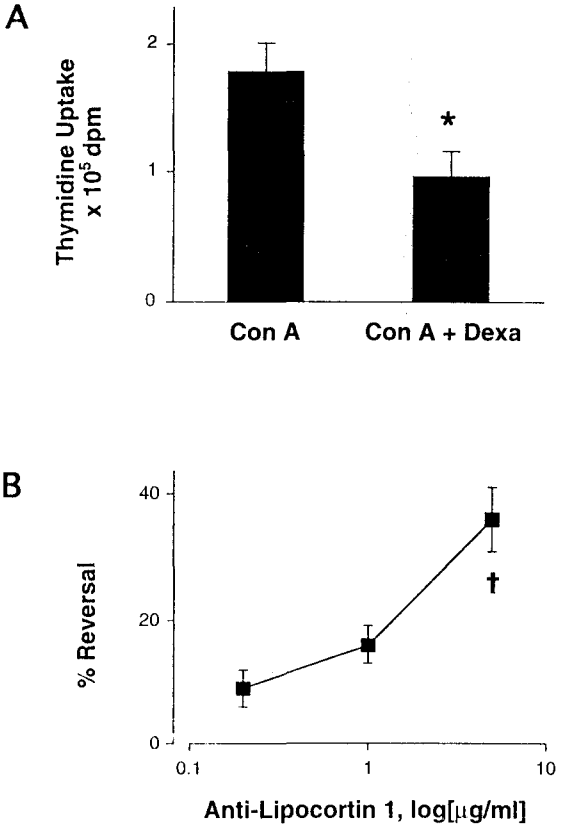

Fig. 2. A) Effect of dexamethasone on ConA-stimulated [^{3}H]-thymidine uptake(dpm). Cells(1×10^5/well) were cultured with 1 μg/ml ConA in the absence or presence of 50nM dexamethasone(n=7). *, P<0.05 vs. ConA-stimulated thymidine uptake without dexamethasone. B) Reversal(%) of the suppressive effect of dexamethasone on ConA-stimulated thymidine uptake by the addition of Anti-LC1(n=7). Cells were cultured with 1 μg/ml ConA, 50nM dexamethasone, and 0, 0.24, 1.2, or 6 μg/ml of Anti-LC1. †, P<0.05 vs. dexamethasone-suppressed thymidine uptake without Anti-LC1.

pressive effect of dexamethasone.

Effect of Anti-LC1 on dexamethasone-induced suppression of ConA-stimulated NO production

Nitrite concentration in the culture medium of unstimulated splenic leukocytes was undetectable (<1 μM). The stimulation of the cells with ConA increased the concentration of nitrite in culture medium to 4.55±0.92 mM. The addition of dexamethasone (50nM) reduced nitrite concentration to 1.40±0.43 μM(P<0.05) which was 31±4% of ConA- stimulated value(Fig. 3). The NO production suppressed by dexamethasone was also significantly recovered by the concomitant addition of Anti-LC1. Anti-LC1 at a concentration of 0.24, 1.2, and 6 μg/ml increased the production of nitrite to 65±11%, 73±14%, 84±13% of ConA-stimulated value, respectively. These represent the reversal of dexamethasone effects by 49±16%, 61±20%, 77±19%, respectively.

DISCUSSION

This study demonstrated that a polyclonal Anti-LC1 reversed at least in part the suppressive effect of dexamethasone on NO generation and proliferation of rat splenic leukocytes in response to ConA. These observations reinforce the concept that lipocortin 1 mediates the anti-inflammatory action of glucocorticoids.

Lipocortin 1 is an endogenous protein with calcium and phospholipid binding properties, which is induced by glucocorticoids(Goulding et al, 1990a & b). The protein has been proposed as a putative mediator of the anti-inflammatory action of glucocorticoids. Following cloning and sequencing, human recommbinant lipocortin 1 itself or a truncated 1-188 fragment is recognized to possess many anti-inflammatory properties: for example, addition to the tissues in vitro

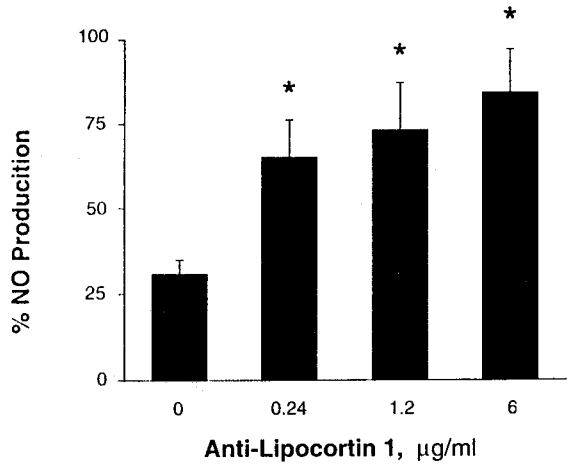

Fig. 3. Reversal of suppressive effect of dexamethasone on ConA-stimulated NO production by the addition of Anti-LC1(n=9). Cells were cultured with 1 μg/ml ConA, 50nM dexamethasone, and 0, 0.24, 1.2, or 6 μg/ml of Anti-LC1. Ordinate represents % of NO production by ConA stimulation without the addition of dexamethasone or Anti-LC1. *, $P<0.05$ vs. dexamethasone-suppressed NO production without Anti-LC1.

leads to rapid suppression of eicosanoid generation(Cirino & Flower, 1987; Cirino et al, 1987) systemic or local administration reduces polymorphonuclear leukocytes migration induced by either interleukin-1(Perretti et al, 1993a & 1993b) or polyacrylamide gel(Errasfa & Russo-Marie, 1989) injection in mice. Local administration of lipocortin 1 also inhibited carrageenin-induced paw edema(Cirino et al, 1989) and cytokine-induced fever in rats(Carey et al, 1990). Some, if not all, of anti-inflammatory actions of lipocortin 1 have been attributed to its inhibition of phospholipase A_2(PLA_2) activity(Flower & Rothwell, 1994). Inhibition of PLA_2 reduces the conversion of membrane pholpholipids into free arachidonic acid, thus, ultimately results in a decreased formation of arachidonic acid-derived mediators of inflammation such as the prostaglandins and leukotriens.

The reversal of dexamethasone effect by Anti-LC1 indicated that glucocorticoids promoted not only the synthesis but also the secretion of lipocortin 1 from cells. Interestingly, lipocortin 1 does not have a signal sequence. However, it can apparently be released from the intracellular compartment to the extracellular cell surface(Croxtall & Flower, 1992) and may occupy high affinity binding sites on the surface of some cells(Goulding et al, 1990b).

The involvement of lipocortin 1 in glucocorticoid induced growth arrest has been described in cultured cells like A549, a human adenocarcinoma cell line (Croxtall & Flower, 1992; 1994; Croxtall et al, 1993). Croxtall & Flower(1992) demonstrated that 5 μg/ml of a neutralizing Anti-LC1 monoclonal antibody reversed the suppression of cell proliferation produced by an EC_{50} concentration of dexamethasone. In our study, dexamethasone suppressed ConA-induced cell proliferation in rat splenic leukocytes with the magnitude which was typical of that observed in other glucocorticoid-sensitive cell lines(Fig. 1 & 2A). However, the addition of polyclonal Anti-LC1 in doses up to 6 μg/ml to the culture medium only partially reversed(about 35%) dexamethasone effect (Fig. 2B) while comparable dose of Anti-LC1 almost completely reversed it in the studies by Croxtall & Flower(1992). The cause of the discrepancy between the 2 studies is not clearly understood. It is, however, possibly caused by the different cell systems used in the 2 studies. Unlike A549 cells employed by Croxtall & Flower(1992), splenic leukocytes in our study are composed of mixed cells(lymphocytes 85%, macrophages 12%; Blauer et al, 1991). Thus, our results represent the outcome of the responses by the different types of cells in splenic leukocytes which may not respond uniformly to dexamethasone and/or Anti-LC1.

Dexamethasone inhibited NO generation by rat splenic leukocytes and Anti-LC1 antibody also reversed this inhibitory activity(Fig 3). It agrees with a recent finding(Wu et al, 1995) showing that the pretreatment of Anti-LC1 ameliorates the inhibition by dexamethasone of the expression of inducible NO synthase in J774.2 macrophages. Since the suppressive effect of dexamethasone on both of cell proliferation and NO generation seem to be mediated by lipocortin 1, one can suspect that a causality exists between the two effects in the present system. As shown in Fig. 2B & 3, the effects of Anti-LC1 on the two processes exhibit quite different dose-response relationships. The addition of Anti-LC1 at the concentration of 0.24 μg/ml reversed about 50% of dexamethasone effect and at 6 μg/ml almost abolished the effect(Fig 3). In addition, it is known that large amounts of NO produced by activated macrophages suppress lymphocyte proliferation and cytokines secreted by activated lymphocytes regulates NO synthesis by macrophages(Moilanen & Vapaatalo,

1995). Thus, there is a possible interaction between the ConA-induced cell proliferation and NO generation processes, and dexamethasone probably modulates the two processes independently.

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