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Regulatory effects of deoxycholic acid, a component of the anti-inflammatory traditional Chinese medicine Niuhuang, on human leukocyte response to chemoattractants

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Abstract

Niuhuang is a commonly used Chinese traditional medicine with immunoregulatory and anti-inflammatory properties. Deoxycholic acid (DCA) is a major active constituent of Niuhuang. The reaction of human leukocytes to chemoattractants is an important part of the host immune response and also plays a crucial role in the development of inflammation. We, therefore, investigated the *in vitro* effects of DCA on human monocyte and neutrophil responses to classic chemoattractants [fMet-Leu-Phe (fMLP), complement fraction 5a (C5a)], CC chemokine [monocyte chemoattractant protein-1 (MCP-1/CCL2)], and/or CXC chemokines [stromal cell-derived factor-1 (SDF-1α/CXCL12), interleukin-8 (IL-8/CXCL8)]. The results showed that DCA significantly inhibited fMLP-induced monocyte and neutrophil chemotaxis and calcium mobilization, and also blocked the binding of [^{3}H]fMLP and anti-formyl peptide receptor (FPR) monoclonal antibodies (mAb) to the cells. The inhibitory effects of DCA on calcium mobilization and anti-FPR-mAb binding to the receptor could be abrogated by washing DCA out of the cell suspension, suggesting that DCA blocked fMLP receptors *via* a steric hindrance mechanism, not *via* receptor internalization. DCA had no significant inhibitory effects on MCP-1-, SDF-1α-, or C5a-induced monocyte function, or C5a- or IL-8-induced neutrophil function. Taken together, our experimental results suggest that blockade of fMLP receptors may contribute to the anti-inflammatory effects of traditional medicine containing DCA.

Keywords: Deoxycholic acid (DCA); Human leukocyte; Chemokine/chemoattractant; Chemotaxis; Calcium mobilization; Ligand–receptor binding

1. Introduction

Biliary products, including Niuhuang (ox gallstone) and bear bile, have been used in China and other Asian countries as therapeutics for thousands of years. The first descriptions of Niuhuang appeared in *Shen Nong Ben Cao Jing* (the Divine Farmer's Herbal Classic, 22–250 AD, the earliest monograph on medicinal materials in China). Among the 365 drugs listed in this book, Niuhuang was recorded as top grade. Under the entry of Niuhuang in *Ben Cao Gang Mu* (Compendium of Materia Medica, 1590), it is indicated for treatment of ''smallpox, madness and delirium''. Other major references in Chinese medicine mention the use of Niuhuang in coma and delirium due to febrile diseases, epileptic convulsion caused by high fever, convulsions in infants, ulcerative gingivitis, retropharyngeal abscess, aphthous stomatitis, large carbuncle, and furuncle [1]. A number of pharmacological and clinical studies suggested that the therapeutic value of Niuhuang includes protection against an irregular beating pattern of myocardial cells [2], and treatment of chronic liver diseases [3]. Niuhuang also was reported to possess sedative,

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Abbreviations: C5a, complement fraction 5a; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; FBS, fetal bovine serum; fMLP, fMet-Leu-Phe; FPR, formyl peptide receptor; Fura-2, 1-[2-(5-carboxyoxazol-2-yl)-6-amino-benzofuran-5-oxy]-2-(29-amino-59-ethylphenoxy)-ethane-*N*,*N*,*N*^{9},*N*^{9}-tetraacetic acid pentaacetoxy methyl ester; IL-8, interleukin-8; mAb, monoclonal antibodies; MCP-1, monocyte chemoattractant protein-1; MFI, mean fluorescence intensity; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; PBMC, peripheral blood mononuclear cell; SDF-1α, stromal cell-derived factor-1.

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anti-spasmodic, anti-pyretic, cardiotonic, hypotensive, anti-convulsive, analgesic, anti-inflammatory, and immunoregulatory effects [4–6]. It has been shown that bile acids (DCA and cholic acid) are major active components of traditional Chinese drugs and formulae in which biliary products are an ingredient [1,7–10]. In addition to the potential transcriptional down-regulation of cytokine gene expression by the bile acid nuclear receptor FXR [11], we hypothesized that bile acids may interfere with chemoattractants and their receptors, thus further augmenting the anti-inflammatory efficacy of bile acids.

Chemoattractants such as chemokines, complement components (C5a), and bacterial products (fMLP) are a group of small molecular weight peptides that, in a concentration-dependent manner, induce directed leukocyte migration *in vitro* and *in vivo*. Leukocyte responses to chemokines and chemoattractants form the first line of host defense to invading microbial agents, and the resultant leukocyte infiltrate plays a crucial role in the development of inflammation [12]. Antagonists of the chemokine and chemoattractant–receptor interaction interfere with leukocyte migration and inflammatory reactions [13]. We previously reported that CDCA, a primary human bile acid, is a selective endogenous antagonist of fMLP receptors [14]. It was also observed in this laboratory that Qingkailing, an injectable traditional Chinese medicinal preparation with Niuhuang as a major component, inhibited the chemotactic response of human monocytes to certain chemoattractants, including fMLP (unpublished data). Furthermore, it has been documented that Niuhuang and its components inhibit leukocyte migration induced by casein [15]. In this paper, we report for the first time that DCA selectively inhibits fMLP functions and regulates chemotaxis of human leukocytes, demonstrating that this is a potential mechanism for the anti-inflammatory functions of DCA.

2. Materials and methods

2.1. Reagents and cells

DCA, obtained from the Sigma, was dissolved in ethanol at 0.1 M. The synthetic formyl peptide fMLP also was purchased from Sigma. [^{3}H]fMLP was purchased from Dupont NEN. CellTiter 96® AQueous was obtained from Promega. Human peripheral blood enriched in mononuclear cells or neutrophils was obtained from normal donors by leukapheresis (Transfusion Medicine Department, Clinical Center, National Institutes of Health, with an approved human subjects agreement). The blood was centrifuged (400 *g*, 30 min, room temperature) through Ficoll–Hypaque (Sigma), and PBMCs collected at the interphase were washed with PBS and centrifuged (400 *g*, 20 min, room temperature) through a 46% isoosmotic Percoll (Pharmacia) gradient. Neutrophils were purified by 3% dextran/PBS sedimentation and were >98% pure. The cells were resuspended in RPMI 1640 medium containing 10% FBS (HyClone) for future use.

2.2. MTS assay

Freshly isolated human monocytes or neutrophils were plated into a 96-microwell plate (Costar) at 1×10^5 cells/well, in the presence of DCA at 200, 100, and 50 μM as the final concentration. For the vehicle control group, medium containing ethanol (0.2%) was used. Cell respiration, as detected by the standard MTS test [16], was measured 12 and 24 hr after co-incubation at 37° in humidified air with 5% CO_2. The effect of DCA on cell respiration was expressed as a percentage of the control group, calculated by the equation: EG × 100/CG, where CG represents the control group value ($OD_{490\ nm}$), and EG the experimental group value ($OD_{490\ nm}$).

2.3. Chemotaxis

Migration of leukocytes was assessed by a 48-well microchemotaxis chamber technique as previously described [17]. Different concentrations of stimulants were diluted in chemotaxis medium [RPMI 1640, 1% BSA (Sigma, A4301), 25 mM HEPES] and placed in the lower compartment of the wells; a 50-μL cell suspension (10^6 cells/mL in chemotaxis medium) was placed in the upper compartment of the well (Neuroprobe). DCA was added to the chemokines and chemoattractants; all groups (including the medium control) contained the same concentration of ethanol (0.2%). The two compartments were separated by a polycarbonate filter (Neuroprobe, 5 μm pore size). After incubation at 37° in humidified air with 5% CO_2 (60–90 min for monocytes, 45–60 min for neutrophils), the filter was removed, fixed, and stained with Diff–Quik (Harlew). The cells on the underside of the membrane were counted at a 200× magnification. The data were expressed as the chemotactic index (CI), which represented the fold increase in the number of cells migrated in response to chemoattractants over the cell response to control medium. Percent inhibition (PI) of chemotaxis was also used, and was calculated by the following formula: $PI = (1 - (EG/CG)) \times 100$, were EG represents the migratory cell number of the experimental group minus spontaneous migration, and CG the migratory cell number of the fMLP positive control group minus spontaneous migration.

2.4. Calcium mobilization

Cellular calcium mobilization was assayed as previously described [18]. Fresh human monocytes or neutrophils suspended in loading buffer (138 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose, and 0.1% BSA, pH 7.4) at a concentration of 10^7 cells/mL were

incubated with 5 μM Fura-2 (Sigma) at 37° for 30 min. The dye-loaded cells were washed twice with 10% FBS in Dulbecco's Modified Eagle's Medium and once with loading buffer, and then were resuspended in fresh loading buffer at a concentration of 5×10^5 cells/mL. Two milliliters of cell suspension was pipetted into quartz cuvettes that were placed in a luminescence spectrometer LS50 B (Perkin-Elmer Limited). Stimulants were added in a volume of 20 μL to the cuvettes at indicated time points. The concentrations reported are the final concentration in the cell suspension. The ratio of fluorescence at 340 and 380 nm wavelengths was calculated using the FL Win Lab (Perkin-Elmer Limited) program. All the samples contained the same concentration of ethanol (0.2%). In another experiment, neutrophils were loaded with Fura-2 and then were incubated with 100 μM DCA at room temperature for 30 min. The cell suspension was divided into two groups. One group was washed three times, and the volume was restored with loading buffer. In the other group, the DCA was not washed out. Cellular calcium mobilization induced by fMLP was recorded and calculated by the method described.

2.5. Binding assay

Human monocytes and neutrophils were suspended in binding medium (RPMI 1640, 1% BSA, 25 mM HEPES). Triplicate samples of cells (1×10^6) were preincubated with DCA (final concentrations were 200, 100, 50, 10, and 5 μM) or medium alone for 10 min at room temperature. All samples contained the same concentration of ethanol (0.2%). A single concentration of radiolabeled fMLP (7.5 nM) or IL-8 (0.12 nM) was added to the samples preincubated with different concentrations of DCA. Samples were incubated at 37° for 20 min with constant mixing. For the [^{3}H]fMLP binding assay, after incubation the cells were washed with ice-cold PBS and collected on Whatman filter discs (GF/C, Whatman International Ltd.) on a 12-well manifold followed by extensive washing with ice-cold PBS. The discs were air-dried at 65°. The radioactivity associated with the cells was measured with a beta counter. For the ^{125}I-IL-8 binding assay, after incubation the cells were centrifuged (14,000 *g*, 30 s, room temperature) through a 10% sucrose/PBS cushion, and the cell-associated radioactivity was measured in a gamma counter. The level of specific binding was determined by subtraction of nonspecific binding (cpm in the presence of 10^{-6} M of fMLP or 1 μg/mL of IL-8) from the total binding (cpm on cells in the absence of unlabeled fMLP or unlabeled IL-8). The percentage of specific binding was calculated by the following equation: % specific binding = 100 × [(binding with DCA − nonspecific binding)/(binding without DCA − nonspecific binding)]. All experiments were performed at least three times, and the results of a representative experiment are presented. All data are expressed as means ± SEM.

2.6. Flow cytometry

The effects of DCA on the binding of anti-FPR, anti-CXCR1, and anti-CXCR2 antibodies to their receptors on human neutrophils were monitored by FACS analysis (courtesy of L. Finch, SAIC Frederick, National Cancer Institute-Frederick Cancer Research and Development Center). Neutrophils (1×10^6) were preincubated with different concentrations of DCA (200, 100, 50, 10, 5 μM) or medium alone (all samples contained the same concentration of ethanol) for 10 min at room temperature, and then were stained with control mAb (MsIgG, Coulter Clone®, 10 μg/mL), or anti-human fMLPR Ab (anti-FPR-mAb, PharMingen, 0.5 mg/mL), or anti-human CXCR1 Ab (PharMingen, 0.5 mg/mL), or anti-human CXCR2 Ab (PharMingen, 0.5 mg/mL), followed by goat anti-mouse IgG (Fc specific)/FITC Ab (Sigma). As a positive control, neutrophils were preincubated with or without 200 μM DCA for 10 min and stained with anti-human CD11b Ab (PharMingen, 0.5 mg/mL). Stained cells were analyzed on an EPICS profile (Coulter Corp.). Data are expressed as mean fluorescence intensity (MFI). The PI of anti-FPR-mAb binding in the presence of different concentrations of DCA was calculated using the MFI values, by the formula: $\mathrm{PI} = (1 - (\mathrm{EG}/\mathrm{CG})) \times 100$, where EG represents the MFI of the DCA-treated samples minus the MFI of the control isotype mAb samples, and CG the MFI of medium-treated (anti-FPR-mAb positive control) samples minus the MFI of control isotype mAb samples.

2.7. Statistical analysis

All experiments were performed at least three times, and the results of a representative experiment are presented. All data are expressed as means ± SEM. The significance of the difference between experimental and control groups was analyzed with a Student's *t*-test.

3. Results

3.1. Effects of DCA on human leukocyte respiration

DCA can be used at millimolar concentrations (0.1–0.5% or 2.55–12.7 mM) as a component of the detergent washing buffer or the lysis buffer. To rule out the possibility that the effects of DCA at micromolar concentrations are due to cell lysis, we co-incubated DCA with human monocytes and neutrophils for 12 and 24 hr. A standard cell respiration (MTS) assay showed that DCA is not cytotoxic at 200 μM, the highest concentration used in this study, during the observation time period. Therefore, the effects of DCA are not due to its lytic property or general cell toxicity (Fig. 1A and B).

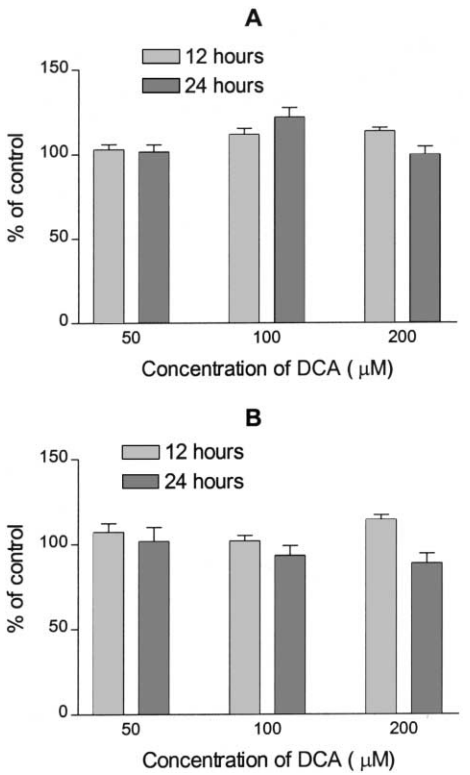

Fig. 1. Effect of DCA on human monocyte (A) and neutrophil (B) respiration. Cells were plated into 96-well plates at a concentration of 1×10^6/well (200 μL). DCA was added to the cell suspension at a final concentration of 50, 100, and 200 μM. After incubation for 12 and 24 hr at 37° in humidified air with 5% CO_2, 20 μL of MTS/PMS solution was added into each well. One hour later, $OD_{490\ nm}$ was determined by a microwell plate reader. The effect of DCA on the cells is expressed as a percentage of the control group as described in Section 2. Data are expressed as means ± SEM ($N = 3$). Even up to 200 μM, DCA did not inhibit monocyte and neutrophil respiration (compare with the control, $P > 0.05$).

3.2. Effects of DCA on human leukocyte migration induced by chemoattractants

First we studied the effects of DCA on human monocyte migration induced by classic chemoattractants (C5a and fMLP), CXC chemokine (SDF-1α), and CC chemokine (MCP-1). As shown in Fig. 2A, at a concentration of 200 μM, DCA significantly inhibited the chemotactic effect of fMLP ($P < 0.001$). In contrast, DCA did not inhibit chemotaxis induced by MCP-1, SDF-1α, or C5a and did not suppress spontaneous migration significantly ($P > 0.05$). The inhibitory effects of DCA on fMLP-induced monocyte migration appeared to be concentration-dependent, with an IC_{50} value of about 100 μM (Fig. 2C).

We next examined the effects of DCA on human neutrophil migration induced by classical chemoattractants (C5a and fMLP) and the CXC chemokine (IL-8). Again, DCA inhibited fMLP-induced neutrophil chemotaxis significantly ($P < 0.001$) in a concentration-dependent manner. The IC_{50} value of DCA inhibition of fMLP-induced neutrophil migration appeared below 100 μM. In contrast, DCA did not inhibit C5a- or IL-8-induced migration and spontaneous migration (Fig. 2B and D).

3.3. Effects of DCA on human leukocyte calcium flux evoked by chemokines and chemoattractants

Fura-2-loaded human monocytes demonstrated a clear and transient calcium flux after stimulation with fMLP, MCP-1, or C5a. When DCA (25 μM) was added before stimulation with these agonists, only the fMLP-evoked calcium flux was inhibited. DCA attenuated fMLP-evoked monocyte calcium mobilization in a concentration-dependent fashion (Fig. 3A). On the other hand, DCA had very little influence on MCP-1- and C5a-evoked monocyte calcium mobilization. In human neutrophils, DCA attenuated calcium mobilization induced by fMLP in a concentration-dependent manner. With a concentration of 25 μM, DCA completely abrogated calcium mobilization induced by fMLP, but DCA did not impact calcium mobilization induced by IL-8 or C5a significantly (Fig. 3B). Furthermore, the inhibitory effects of DCA (100 μM, 30 min) on fMLP-evoked calcium mobilization were reversed by washing (Fig. 4).

3.4. Effects of DCA on [^{3}H]fMLP and ^{125}I-IL-8 binding to human leukocytes

Chemoattractants mediate their biological function by binding to their receptors on the cell membrane. We examined the effects of DCA on the binding of radiolabeled fMLP and IL-8 to leukocytes. As shown in Fig. 5, DCA blocked [^{3}H]fMLP binding to the monocytes (panel A) and neutrophils (panel B) in a concentration-dependent fashion (cpm of 100% specific binding was 16,900). No obvious influence was observed by DCA on the binding of IL-8 to the neutrophils (Fig. 5C) (cpm of 100% specific binding was 27,200).

3.5. Effects of DCA on FPR, CXCR1, and CXCR2 specific antibody binding

To confirm the results of the ligand binding studies, we evaluated the effects of DCA on antibodies specific to FPR, CXCR1, and CXCR2 binding with the cells. Preincubation of neutrophils with DCA at concentrations ranging from 5 to 200 μM resulted in a concentration-dependent decrease of anti-FPR-mAb binding to the cells. The percent inhibition by 200 μM DCA was 69.5%, whereas 5 μM DCA resulted in only 6.38% inhibition (Table 1, Fig. 6A). In contrast, up to 200 μM DCA only slightly inhibited anti-CXCR1 and anti-CXCR2 antibody binding to neutrophils (Fig. 6B and C). DCA did not influence isotype-matched antibody (anti-CD11b antibody) binding (Fig. 6D), excluding the possibility that the effects of DCA were based on its interaction with the antibody. The reversibility of the

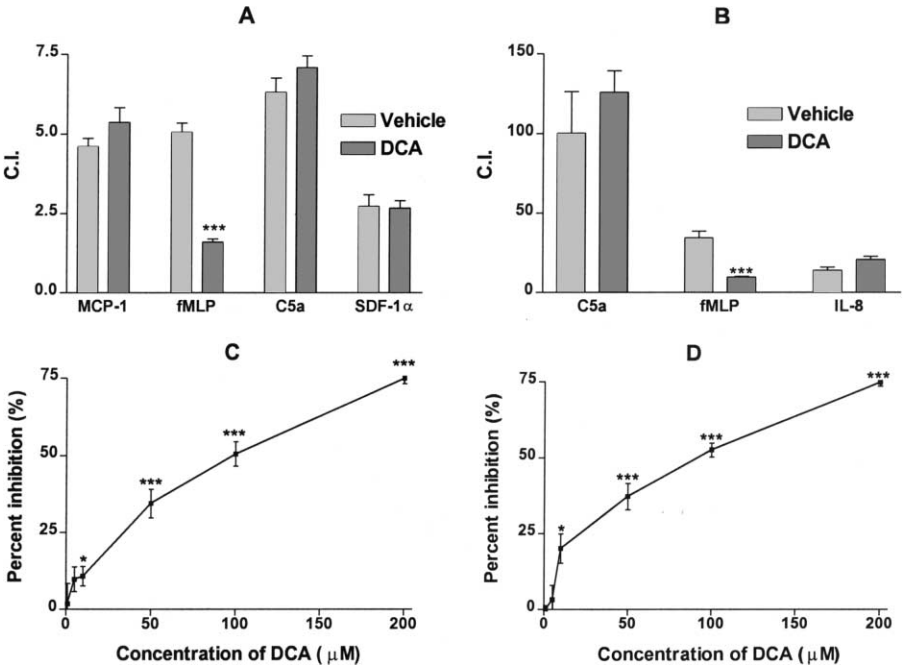

Fig. 2. Effect of DCA on human monocyte and neutrophil migration induced by chemoattractants. Chemoattractants were placed in the lower wells of chemotaxis chambers, with the desired concentration of DCA. Cell suspensions were placed in the upper wells. The upper and lower wells were separated by polycarbonate filters. After incubation, the cells that migrated across the filters were stained and counted. For a better demonstration, data are expressed as CI or percent inhibition (%). (A) Effects of DCA (200 μM) on monocyte migration induced by MCP-1 (10 ng/mL), fMLP (10^{-8} M), C5a (10^{-8} M), and SDF-1α (100 ng/mL). (B) Effects of DCA (200 μM) on neutrophil migration induced by C5a (10^{-8} M), fMLP (10^{-8} M), and IL-8 (100 ng/mL). (C) Concentration-dependent inhibition of DCA on fMLP (10^{-8} M)-induced monocyte migration. (D) Concentration-dependent inhibition of DCA on fMLP (10^{-8} M)-induced neutrophil migration. Data are expressed as means ± SEM ($N = 3$) and represent one of three separate experiments with similar results. The single asterisk (∗) and the triple asterisks (∗∗∗) indicate $P < 0.05$ and $P < 0.001$ respectively, compared with the control group.

Table 1
Anti-FPR-mAb binding to neutrophils

DCA (μM)	MFI	PI (%)
0	13.5	0
5	12.7	6.4
10	11.6	15.2
50	8.9	36.8
100	7.6	46.7
200	4.8	69.5
Control	1	

Neutrophils were preincubated with vehicle alone or with DCA at different concentrations at room temperature for 10 min. The cells were stained with anti-FPR-mAb or isotype-matched mAb (control), followed by FITC-conjugated anti-mouse IgG antibody. MFI, mean fluorescence intensity; PI, percent inhibition of MFI. This experiment was performed three times.

inhibitory effect of DCA on FPR was demonstrated by completely washing DCA out of the cell suspension and restoring the anti-FPR antibody binding to the cells (Fig. 6A).

4. Discussion

Chemoattractants include classical chemoattractants (such as bacterial-derived *N*-formyl peptides [19], complement fragment peptides C5a and C3a [20], lipid molecules such as leukotriene B_4 [21], and platelet-activating factor [22]) and a class of 8- to 15-kDa chemotactic cytokines (chemokines) that have characteristic conserved cysteine residues [23]. Chemoattractant receptors belong to the family of seven transmembrane domain G protein-coupled receptors. By binding to their receptors, chemoattractants produce their biological effects, which are (a) directing the trafficking of phagocytic leukocytes, and (b) activation of leukocytes with the generation of superoxide anions and release of granule contents that enable leukocytes to serve as the first-line host defense against invading microorganisms [12]. However, inappropriate or excessive activation of phagocytes by chemoattractants is often a major cause of tissue damage. A number of diseases, ranging from HIV-1 infection [24] and solid tumor growth [25] to acute and chronic inflammation [26], have been shown to involve chemoattractant activity. Therefore, the discovery and design of chemoattractant inhibitors and antagonists may yield drugs with significant therapeutic benefit [27–29].

Among all the chemoattractants, fMLP is unique in many aspects. It is one of the first identified leukocyte chemoattractants. The prototype *N*-formyl peptide, CHO-fMLP, contains only three amino acids. fMLP is also one of the most potent peptide chemoattractants, being able to induce all major phagocyte functions. Thus far, the high-affinity fMLP receptor is the only receptor that recognizes

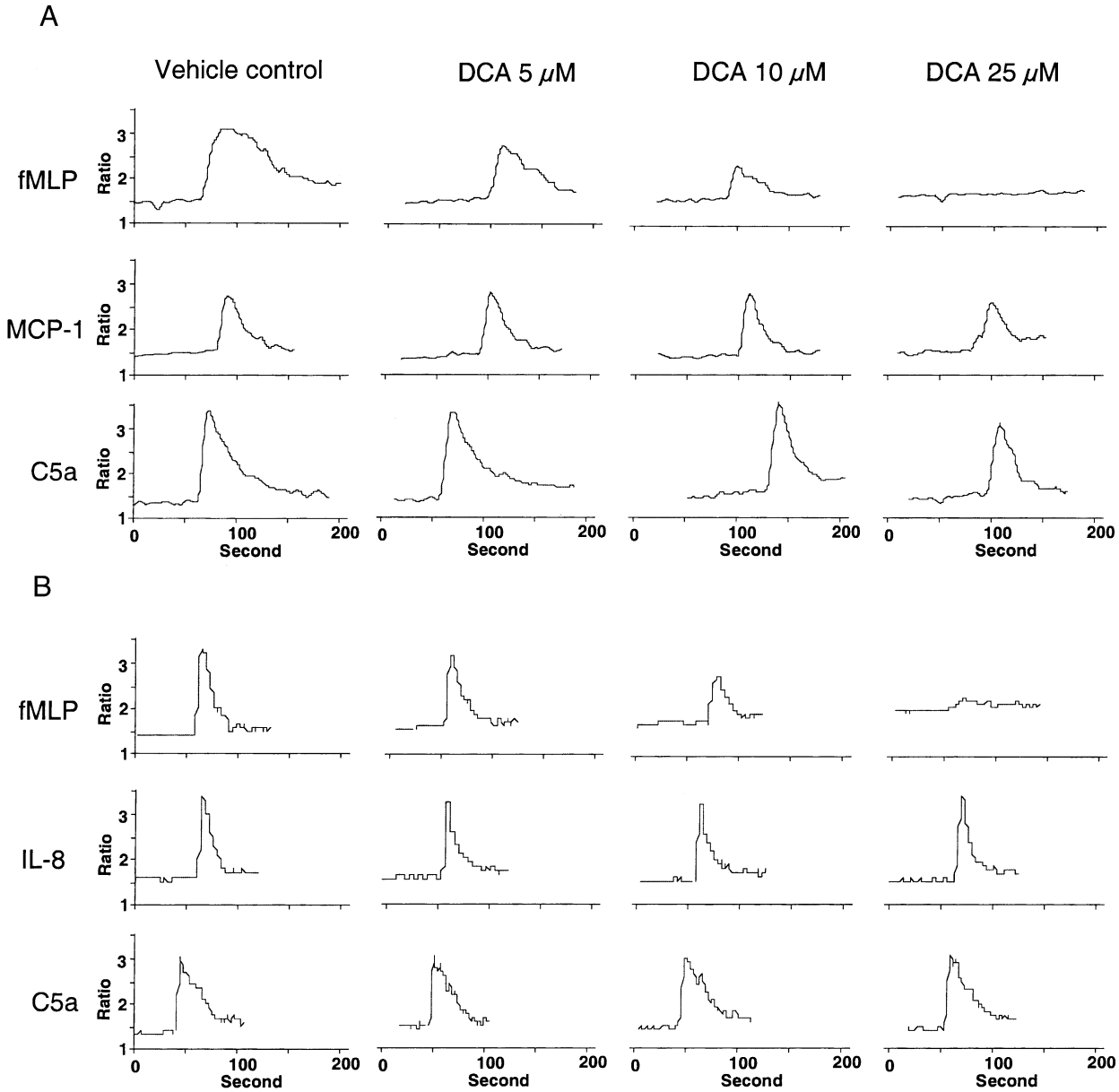

Fig. 3. Effect of DCA on human monocyte and neutrophil calcium mobilization induced by chemoattractants. Human monocytes and neutrophils were loaded with Fura-2 and then were stimulated with DCA at the indicated concentration, followed by stimulation with fMLP (final concentration 10^{-9} M), MCP-1 (for monocytes, 100 ng/mL), IL-8 (for neutrophils, 100 ng/mL), or C5a (10^{-9} M). The ratio of fluorescence at 340 and 380 nm wavelengths was recorded and calculated using the FL Win Lab program. Labels on the top of the figure represent the final concentration of DCA and labels on the left of the panel represent the chemoattractant stimulants. (A) Human monocytes, and (B) human neutrophils. This experiment was performed three times.

a low concentration of exogenous chemoattractant, the bacterially derived *N*-formyl peptides [30,31]. The main responses elicited by fMLP in granulocytes include cell polarization, generation of reactive oxygen species, production of arachidonic acid metabolites, and release of lysosomal enzymes [31]. As a potent proinflammatory peptide, fMLP also potentially contributes to the inflammatory damage. fMLP has been implicated in the initiation and perpetuation of inflammatory bowel disease [32]. Colonic instillation of fMLP has been used to induce an animal model of mucosal inflammation [33]. Furthermore, fMLP stimulates proinflammatory cytokine gene expression in leukocytes [34], and may play a causative role in inflammation and autoimmune diseases associated with proinflammatory cytokines. Therefore, inhibition of the interaction of fMLP with its receptor may provide a new stratagem for modulating infectious microbial inflammatory diseases. A considerable effort has been made to identify fMLP antagonists. So far, a number of fMLP inhibitors have been identified, such as spinorphin [35], taxol [36], losartan [37], sulfasalazine [38], and ryanodine [39].

In this study, we demonstrated that DCA selectively inhibits fMLP-induced human monocyte and neutrophil

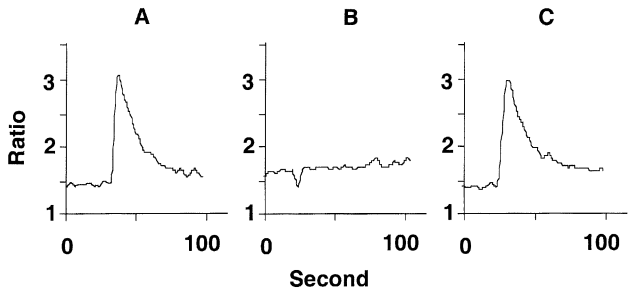

Fig. 4. Effect of pulse exposure of DCA on calcium mobilization of chemoattractant-stimulated human neutrophils. For control, neutrophils loaded with Fura-2 were stimulated with fMLP (10^{-9} M) without treatment with DCA (A). Fura-2-loaded human neutrophils were pretreated with 100 μM DCA at room temperature for 30 min. The cells were divided into two groups. In one group (B), the cell suspension was stimulated with fMLP (10^{-9} M) in the presence of DCA. In the other group (C), cells were washed three times with PBS and then restored to volume with loading buffer and stimulated with fMLP (10^{-9} M). The ratio of fluorescence at 340 and 380 nm wavelengths was recorded and calculated using the FL Win Lab program. This experiment was performed three times.

migration and calcium mobilization. This functional inhibition appears to be due to blocking fMLP binding to its receptors on leukocytes. Inhibitory effects of DCA on fMLP-evoked calcium mobilization and anti-FPR-mAb binding to the cells could be abolished after washing out fMLP. We have previously observed that CDCA not only inhibits the effects of fMLP, but also the effects of W peptide (a potent agonist of fMLP receptors that lacks the *N*-formyl structure). CDCA blocked both radiolabeled fMLP and FPR mAb binding to the cells. We hypothesize that CDCA blocks fMLP receptors by steric hindrance [14]. The biological effects of bile acids are highly dependent upon their chemical structure and physicochemical properties [40]. DCA and CDCA are hydrophobic bile acids, and both of them inhibit endotoxin-induced tumor necrosis factor released from leukocytes [41]. In the profile of blockage of fMLP interaction with its receptor, they most likely share the same mechanism.

Variations in DCA efficacy were observed between the different functional cellular assays, e.g. 25 μM DCA completely attenuated fMLP-evoked calcium flux, while 50 μM DCA partially inhibited fMLP-induced chemotaxis. Besides measuring different cellular functions, these assays were performed at different concentrations of cells and fMLP. The individual functional assay conditions were optimized for the individual cellular response being measured such that 10^{-9} M fMLP was needed to induce 1×10^6 cells to calcium flux, while 10^{-8} M fMLP was needed to induce 5×10^4 cells to migrate. It is logical to consider that blockage of FPR by a certain amount of DCA varies with the conditions for individual functional assays.

Although micromolar concentrations of DCA are needed to produce inhibitory effects, this does not negate pathophysiological relevance since higher concentrations of DCA are also achieved *in vivo*. DCA is a secondary human bile acid, which is normally produced in the colon by the actions of intestinal bacteria on primary bile acids [42]. DCA constitutes the major bile acid in the colon with up to 700 μM being measured in fecal water [43–46]. Therefore, the intestinal epithelium can be in contact with high concentrations of DCA. Additionally, fMLP is produced by intestinal bacteria [47], and is present in micromolar concentrations in fecal dialysis fluid *ex vivo* [48]. As both DCA and fMLP exist at high concentrations in the intestine, the inhibitory effect of DCA may play a role in preventing pathological action of fMLP. Further investigation should be undertaken to confirm this hypothesis.

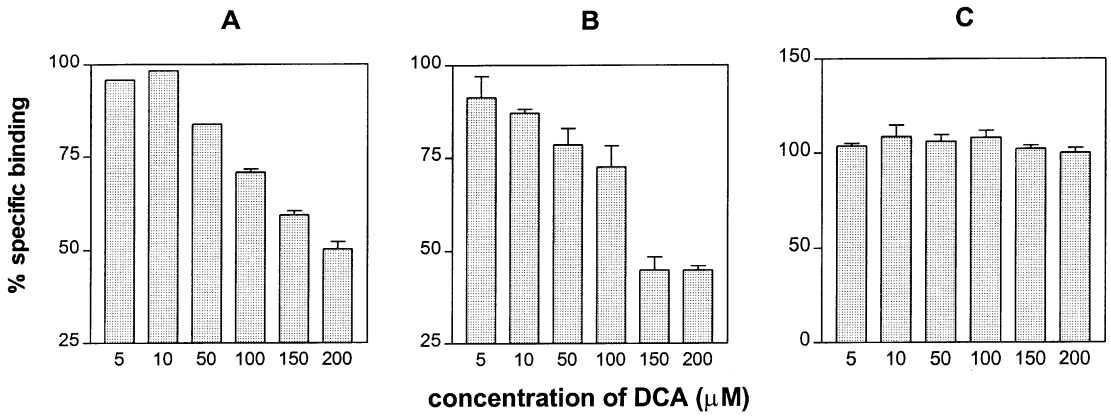

Fig. 5. Effects of DCA on radiolabeled fMLP and IL-8 binding to human leukocytes. Cells were preincubated in triplicate with vehicle alone or with different concentrations of DCA at room temperature for 10 min. Aliquots of cells (1×10^6/200 μL) were incubated with a constant concentration of [^{3}H]fMLP (7.5 nM) or ^{125}I-IL-8 (1 μg/mL) at 37° for 20 min with constant mixing. For the [^{3}H]fMLP binding assay, the cells were then washed with ice-cold PBS and collected on Whatman filter discs. The radioactivity associated with the cells was measured with a beta counter. For the ^{125}I-IL-8 binding assay, after incubation the cells were centrifuged (14,000 *g*, 30 s, room temperature) through a 10% sucrose/PBS cushion, and the cell-associated radioactivity was measured in a gamma counter. The level of specific binding was determined by subtraction of nonspecific binding (cpm in the presence of 10^{-6} M unlabeled fMLP or cpm in the presence of 1 μg/mL of IL-8) from the total binding (cpm in cells with the vehicle). The percentage of specific binding was calculated. (A) fMLP binding to monocytes. (B) fMLP binding to neutrophils. (C) IL-8 binding to neutrophils. Data are expressed as means ± SEM ($N = 3$).

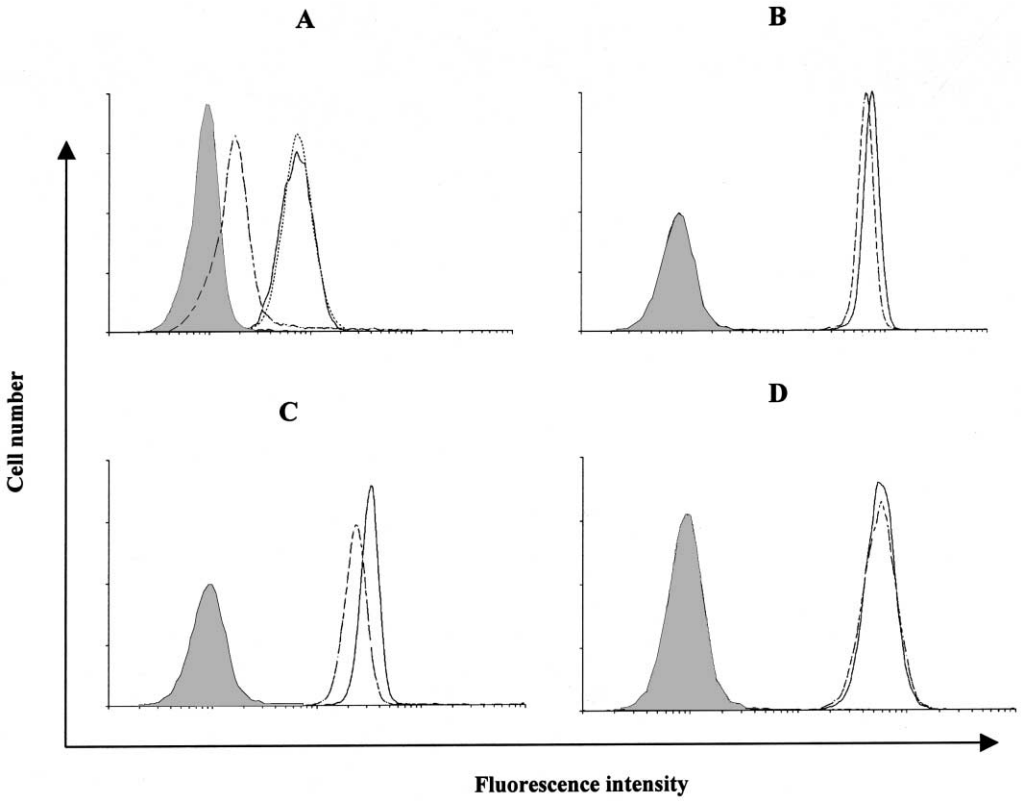

Fig. 6. Effect of DCA on anti-FPR, anti-CXCR1, CXCR2, and anti-CD11b mAb binding to human neutrophils. Neutrophils were stained with control mIgG (shaded area) or specific antibody (—), or pretreated with 200 μM DCA for 10 min at room temperature and then stained with antibody in the presence of DCA (- - -). (A) Cells were stained with anti-FPR-mAb. Additional cells were pretreated with 200 μM DCA and then were washed three times before staining with anti-FPR-mAb (. . .). (B) Cells were stained with anti-CXCR1 mAb. (C) Cells were stained with anti-CXCR2 mAb. (D) Cells were stained with anti-CD11b mAb. This experiment was performed three times.

Unlike other traditional Chinese herbal medicines, Niuhuang and bear bile are obtained from animals. The production is limited, and the price is extremely high. For practical purposes and for conservation reasons, it is desirable to find an acceptable substitute to the animal biliary products used in traditional medicines. Some efforts have been made. For example, pig bile shares properties (including anti-inflammation) similar to those of bear bile and has been proposed as a substitute for bear bile [5]. Alternatively, identification of the active agents in biliary products and their pharmacological properties may eliminate the need for any animal source. Our data suggest that one of the mechanisms of the anti-inflammatory effects of Niuhaung may be the blockade of fMLP receptors. DCA, a more easily accessible chemical compound than animal biliary products, could perhaps be an effective substitute for Niuhuang in the future treatment of bacterial-induced inflammation.

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