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Regulation of Immune Complexes Binding of Macrophages by Pectic Polysaccharide from *Bupleurum falcatum* L.: Pharmacological Evidence for the Requirement of Intracellular Calcium/Calmodulin on Fc Receptor Up-regulation by Bupleuran 2IIb

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Abstract

The pectic polysaccharide, bupleuran 2IIb, up-regulates Fc-receptor (FcR) expression on peritoneal macrophages in a dose-dependent manner. The intracellular signal transduction by bupleuran 2IIb leading to the expression of FcR was studied.

Neither the protein kinase C (PKC) inhibitor, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine dihydrochloride, nor the structurally distinct PKC antagonist, calphostin C, inhibited bupleuran 2IIb-induced up-regulation of FcR, whereas two direct activators of PKC, L-α-1-oleoyl-2-acetyl-*sn*-3-glycerol and *N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulphonamide were unable to up-regulate the expression of FcR. The protein kinase A (PKA) inhibitor, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride also did not inhibit bupleuran 2IIb-induced up-regulation of FcR. Fluorescence image analysis using the calcium-sensitive dye, Fura-2, demonstrated that bupleuran 2IIb induced a rapid increase in intracellular levels of calcium (Ca^{2+}). When macrophages were treated with calcium antagonist, 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride, bupleuran 2IIb-induced up-regulation of FcR was inhibited in a dose-dependent manner. The bupleuran 2IIb-induced up-regulation of FcR was also blocked by two structurally distinct calmodulin antagonists, trifluoperazine and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride. Furthermore, elevation of intracellular Ca^{2+} using the calcium ionophore, A23187, led to up-regulation of the FcR expression in a dose-dependent manner.

These results suggest that bupleuran 2IIb induces the up-regulation of FcR on macrophages by a mechanism dependent on an increase in intracellular Ca^{2+} followed by activation of the calmodulin, but not by a PKC or PKA pathway.

The receptor for Fc portion of immunoglobulin (FcR) on macrophages plays an important role in the host defence system; clearance of immune complexes, antibody-dependent cell-mediated cytotoxicity (ADCC), tumouricidal action and prostaglandin release (Larrick et al 1980; Leslie 1980; Passwell et al 1980; Frank 1983). Recently, we found that the acidic pectic polysaccharide, bupleuran 2IIb, from *Bupleurum falcatum* L., enhanced immune complex binding to murine peritoneal macrophages based on up-regulation of the expression of FcRs (Matsumoto et al 1993). However, the biochemical mechanisms involved in signal transduction leading to the up-regulation of FcR by bupleuran 2IIb has been unclear. We have previously reported (Matsumoto et al 1990) that 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a potent protein kinase C (PKC) activator (Castagna et al 1982), enhances the immune complex binding of macrophages as bupleuran 2IIb does. It is possible that PKC may be involved in FcR up-regulation by bupleuran 2IIb. For these reasons, we investigated the effects of PKC regulators and calcium regulators on FcR up-regulation by bupleuran 2IIb.

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Materials and Methods

Materials

The roots of *Bupleurum falcatum* L. were obtained from Uchida Wakan Yaku (Tokyo, Japan). Mouse glucose oxidase-anti-glucose oxidase complex (GAG) was obtained from ICN Immuno Biologicals (Lisle, IL). Foetal bovine serum (FBS) was obtained from Cell Culture Laboratories (Cleveland, OH). Fura-2 acetomethoxyester (Fura-2-AM) was from Dojin (Kumamoto, Japan). Penicillin, streptomycin and amphotericin B were from Flow Laboratories (Irvine, UK). Calphostin C was purchased from Kyowa Medix (Tokyo, Japan). Trifluoperazine dihydrochloride and 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) were purchased from Sigma (St Louis, MO). *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W7) and *N*-(6-aminohexyl)-1-naphthalenesulphonamide hydrochloride (W5) were purchased from Biomel Res. Lab. Inc. (Campus Drive Plymouth Meeting, PA). 1-(5-Isoquinolinylsulphonyl)-2-methylpiperazine dihydrochloride (H-7), *N*-[2-(methylamino)ethyl)]-5-isoquinolinesulphonamide dihydrochloride (H-8), *N*-(2-aminoethyl)-5-isoquinolinesulphonamide dihydrochloride (H-9), *N*-(2-guanidinoethyl)-5-isoquinolinesulphonamide hydrochloride (HA1004) and *N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulphonamide (SC-9) were from Seikagaku Kogyo (Tokyo,

Japan). A23187 was from Calbiochem (La Jolla, CA). L-α-1-Oleoyl-2-acetyl-*sn*-3-glycerol (OAG) was from Avanti Polar Lipids, Inc. (Birmingham, AL).

Preparation of FcR expression-enhancing polysaccharide (bupleuran 2IIb)

The acidic pectic polysaccharide, bupleuran 2IIb, was purified by precipitation with cetyltrimethylammonium bromide and chromatography on DEAE-Sepharose CL-6B from BR-1 as described previously (Yamada et al 1989, 1991).

Preparation of macrophage monolayer

The preparation of the macrophage monolayer was described previously (Matsumoto et al 1990). Briefly, male SPF ICR mice (6–8-weeks old) (SLC, Japan) were injected intraperitoneally with 2 mL thioglycollate medium 3 days before the preparation of peritoneal exudate cells (PEC). The mice were killed and the peritoneal cavities were washed with Hanks' balanced salt solution supplemented with 12·5 mM HEPES, pH 7·2 (HBSS) containing 5 units mL^{-1} heparin (Novo, Copenhagen, Denmark). The wash-outs were centrifuged at 100 *g*, and washed once with HBSS. The washed cells were resuspended at a concentration of $1·0 \times 10^6$ PEC mL^{-1} in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FBS, 12·5 mM HEPES (pH 7·35), 100 int. units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 0·25 μg mL^{-1} amphotericin B. The PEC suspension was dispensed into a sterile flat-bottomed 96-well micro culture plate. After the plate was incubated at 37°C in a moist 95% air–5% CO_2 atmosphere for 2 h, the non-adherent cells were removed by washing with HBSS and the determination of Fc receptor function was performed using the remaining adherent cells as macrophages.

Determination of Fc-receptor-mediated immune complex binding

Determination of Fc-receptor-mediated immune complex binding was performed according to the method reported previously (Matsumoto et al 1990). Briefly, macrophages were cultured in the absence or presence of test samples for 15 h at 37°C. Thereafter, the macrophage monolayer was washed with phosphate-buffered saline containing 1% BSA (PBS-1% BSA), then 100 μL ice-cold glucose oxidase-anti-glucose oxidase complex (GAG, a model immune complex) solution was added to each well and incubated for 240 min at 4°C; incubation was terminated by aspirating the GAG solution. The macrophages were washed with PBS-1% BSA to remove unbound GAG followed by solubilization in 50 μL 1·0% Nonidet P-40 (NP-40). Reacting fluid consisting of 1 unit mL^{-1} horseradish peroxidase, 50 mg mL^{-1} glucose and 1 mg mL^{-1} 2,2′-azinobis (3-ethylbenzthizoline sulphonic acid) (ABTS) was added to each well (150 μL), and incubated at room temperature (21°C). After 30 min, extinction was measured using a Micro Plate Reader MPR A4 (Tohso, Japan) at 405 nm.

Determination of the effects of intracellular signal transduction inhibitors

To examine the effects of signal transduction inhibitors, the macrophages were pretreated with each signal transduction inhibitor for 30 min, and then cultured with both bupleuran 2IIb (50 μg mL^{-1}) and inhibitor for 15 h.

Analysis of intracellular calcium concentration

Intracellular free calcium (Ca^{2+}) concentration was monitored using the calcium-sensitive indicator dye fura-2 AM. Cells were inoculated onto coverglass and adhered for 2 h. Cells were then washed with HBSS and incubated with 5 μM fura-2AM in HBSS containing 1% BSA at 37°C. After 30 min, the loaded cells were washed with HBSS three times and then RPMI 1640 medium was added. The intracellular Ca^{2+} concentration was measured by a video camera equipped with an ARUGUS-100/Ca system (Hamamatsu Photonics, Shizuoka, Japan) which controls the image acquisition and display. For measurement of intracellular fluorescence, fura-2AM was excited using two excitation wavelengths of 340 and 380 nm. Obtained data were analysed utilizing Image and Analyzing System Software 1 (Ver. 3.59s) (Hamamatsu Photonics).

Statistical analysis of data

Data obtained from experiments are expressed as mean ± s.d. The differences between the control and the treatment in these experiments were tested for statistical significance by Student's *t*-test. A value of $P < 0·05$ was considered statistically significant.

Results and Discussion

Recently, we found that bupleuran 2IIb up-regulated the expression of FcRs on the macrophages, and that this activity was due to enhancement of transcription of both FcγRI and FcγRII genes (Matsumoto et al 1993).

The regulation of intracellular signal transduction pathways in the cellular response to stimulation can be studied by measurement of the intracellular second messengers, determination of the cellular response in the presence of specific inhibitors, and reproducibility of the cellular response by direct stimulation of the signal transduction pathway. To determine whether the PKC activation was involved in FcR up-regulation by bupleuran 2IIb, macrophages were incubated with PKC inhibitors. PKC is known to have two separate functional domains, the catalytic and the regulatory domains, by proteolysis (Inoue et al 1977). The isoquinolinesulphonamide inhibitor, H-7 has a K_i of 6 μM for isolated PKC, and interacts with the catalytic domain of PKC (Hidaka et al 1984). When the macrophages were exposed to increasing doses of H-7 for 30 min and then incubated with H-7 and bupleuran 2IIb (50 μg mL^{-1}) for 15 h, H-7 had no effect on the up-regulation of FcR by bupleuran 2IIb (data not shown). Three other isoquinolinesulphonamide PKC inhibitors, H-8, H-9 and HA1004, which are less potent inhibitors of PKC than H-7 (Hidaka et al 1984), also did not affect the up-regulation of FcR by bupleuran 2IIb (data not shown). The structurally distinct calphostin C, which is a specific inhibitor of PKC with an IC50 value of 50 nM in a cell-free system, has been reported to interact with the regulatory domain of PKC (Kobayashi et al 1989). The effect of calphostin C on up-regulation of FcR by bupleuran 2IIb was examined, but was also ineffective (data not shown). These results suggest that

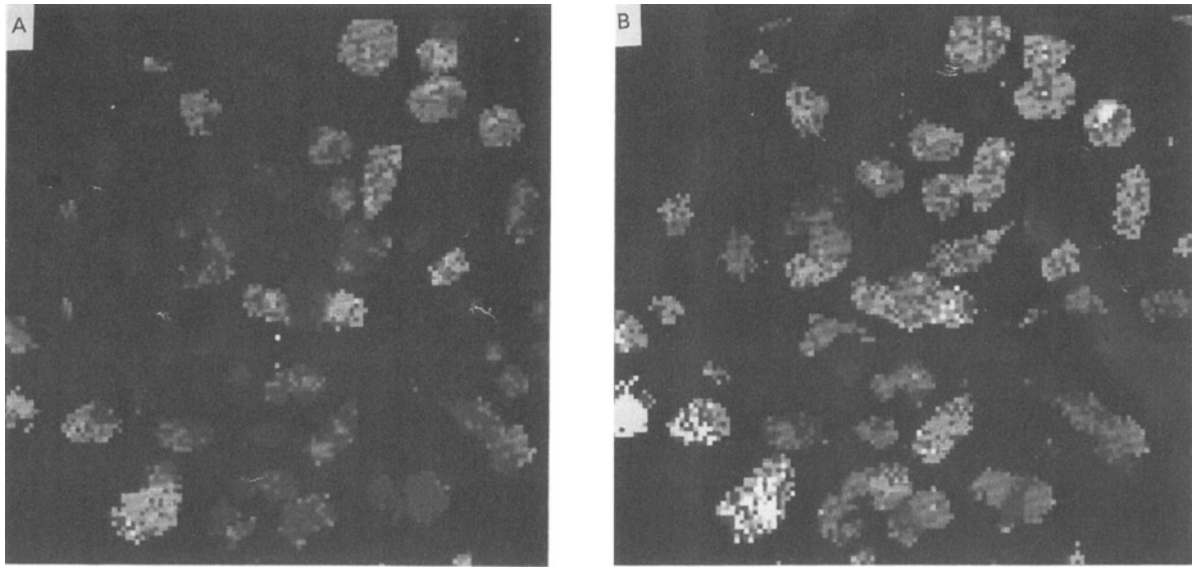

FIG. 1. Fluorescence image analysis of intracellular free calcium concentrations in macrophages, A, just before stimulation; B, 30 s after stimulation. Cells were incubated with fura-2AM (5 μM) and analysed on the ARUGUS-100/Ca system. After a stable baseline was established, the cells were stimulated with bupleuran 2IIb (100 μg mL^{-1}).

bupleuran 2IIb induces the up-regulation of FcR on macrophages by a PKC-independent pathway.

Although H-8 shows relatively potent protein kinase A (PKA)-inhibiting activity than H-7, H-9 and HA1004 (Hidaka et al 1984; Hagiwara et al 1987), H-8 did not affect the up-regulation of FcR by bupleuran 2IIb. Therefore PKA also might not be involved in the up-regulation of FcR by bupleuran 2IIb.

Two PKC activators, OAG, which is a synthetic membrane-permeable diacylglycerol (Mori et al 1982), and SC-9, which is a naphthalenesulphonamide derivative (Ito et al 1986), had no effect on the expression of FcR at concentrations of 100 and 50 μM, respectively (data not shown). These results are in accord with the results on the PKC inhibitors as above, and suggested that PKC may not be involved in FcR up-regulation by bupleuran 2IIb. We have previously reported (Matsumoto et al 1990) that TPA, which is a potent PKC activator, enhances the immune complex binding of macrophages. Our present results also suggest that FcR up-regulation by TPA is due to a PKC-independent mechanism, and that other signal transduction pathways may be required.

It has been reported that intracellular Ca^{2+} may play an important role in the functional responses of macrophages when stimulated by several agents such as interferon-γ and calcium ionophore (Klein et al 1990; Buchmuller-Rouiller & Mauel 1991). Therefore, the effect of bupleuran 2IIb on intracellular Ca^{2+} levels was analysed using the calcium-sensitive indicator dye fura-2AM. The stimulation of the macrophages with bupleuran 2IIb induced a rapid increase in intracellular Ca^{2+} concentrations (Figs 1, 2). This result suggests that changes in intracellular Ca^{2+} are an early event in bupleuran 2IIb-induced signal transduction.

To determine whether the increase of intracellular Ca^{2+} contributes to the FcR up-regulation, the effect of an intracellular Ca^{2+} antagonist, TMB-8, on FcR up-regulation by bupleuran 2IIb was studied. TMB-8 is known to inhibit the release of Ca^{2+} from intracellular storage sites in the endoplasmic reticulum (Chiou & Malagodi 1975; Smith & Iden 1979). TMB-8 inhibited bupleuran 2IIb-induced FcR up-regulation in a dose-dependent fashion from 0 to 25 μM, and 50% inhibition of enhancement of immune complex binding was observed at 10 μM TMB-8 (Table 1). At concentrations of TMB-8 used in this experiment, loss in cell viability was not observed according to the trypan blue dye exclusion test (data not shown). This result suggests that the increase in Ca^{2+} released from an intracellular storage site contributes to up-regulation of FcR.

Because calmodulin can be activated by an increase in intracellular Ca^{2+}, we examined whether inhibition of the calmodulin-dependent process prevented the up-regulation of FcR by bupleuran 2IIb. Trifluoperazine, which is an inhibitor of calmodulin activity (Levin & Weiss 1977), inhibited bupleuran 2IIb-induced FcR up-regulation in a

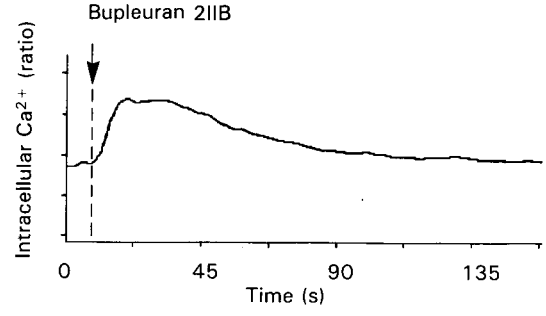

FIG. 2. Changes in intracellular calcium by stimulation of bupleuran 2IIb in macrophages. Cells were incubated with fura-2AM (5 μM) and analysed on the ARUGUS-100/Ca system. After a stable baseline was established, the cells were stimulated with bupleuran 2IIb (100 μg mL^{-1}) at the point indicated (vertical dotted line). Calcium concentrations were indicated by an increase in the fura-2AM fluorescence ratio. Data represent the average change in ratio in a field containing more than 30 cells.

Table 1. The effect of drugs on the bupleuran 2IIb-induced enhancement of GAG binding. Macrophages were treated with varying concentrations of test drugs in the presence or absence of 50 μg mL^{-1} bupleuran 2IIb; after 15 h, GAG-binding activity of macrophages was measured.

Drug	Concn (μM)	GAG binding (± s.d.) (optical density at 405 nm)		S.I.[a]
		With bupleuran 2IIb	Without bupleuran IIb	
TMB-8				
	0	0·41 ± 0·01	0·17 ± 0·00*	2·4
	5	0·29 ± 0·01	0·16 ± 0·01*	1·5
	10	0·22 ± 0·00	0·14 ± 0·01*	1·6
	15	0·20 ± 0·01	0·14 ± 0·00*	1·4
	20	0·14 ± 0·01	0·11 ± 0·01*	1·3
	25	0·10 ± 0·00	0·10 ± 0·00	1·0
Trifluoperazine				
	0	0·41 ± 0·02	0·19 ± 0·01*	2·2
	1	0·40 ± 0·01	0·18 ± 0·00*	2·2
	2	0·40 ± 0·01	0·17 ± 0·01*	2·4
	4	0·26 ± 0·01	0·15 ± 0·00*	1·7
	6	0·14 ± 0·01	0·10 ± 0·00*	1·4
	8	0·09 ± 0·00	0·07 ± 0·01*	1·3
W7				
	0	0·35 ± 0·02	0·18 ± 0·01*	1·9
	10	0·19 ± 0·03	0·16 ± 0·01	1·2
W5				
	0	0·34 ± 0·01	0·19 ± 0·02*	1·8
	10	0·30 ± 0·03	0·19 ± 0·02*	1·6

*$P < 0·01$ compared with corresponding experiment with bupleuran 2IIb. [a] S.I. = Stimulation index, calculated as the ratio of CAG binding with bupleuran 2IIb to binding without bupleuran 2IIb. Each value is the mean of four experiments ± s.d.

dose-dependent fashion from 2 to 8 μM, and 50% inhibition of enhancement of immune complex binding was observed with 4 μM trifluoperazine (Table 1). A structurally distinct calmodulin antagonist, W7 (10 μM), which is a naphthalenesulphonamide competitive inhibitor of enzyme activation by calmodulin (Hidaka et al 1981), also inhibited FcR up-regulation by bupleuran 2IIb treatment, but the markedly less effective antagonist, W5, which is structurally similar to W7, did not (Table 1). The inhibitory effects of trifluoperazine and W7 were not due to loss in cell viability or to cell loss during plate washing (data not shown). These results suggest that Ca^{2+}/calmodulin is an important pathway in the signal transduction leading to up-regulation of FcR by bupleuran 2IIb. To determine whether the rise in the cytosolic Ca^{2+} is essential to up-regulation of FcR, the effect of calcium ionophore A23187 was examined. Macrophages were cultured with various concentrations of A23187 for 15 h. As shown in Fig. 3, A23187 up-regulated the expression of FcR of macrophages in a dose-dependent manner as bupleuran 2IIb did, and reached an almost constant value over 100 nM. When 100 nM A23187 was added to the culture medium, the immune complex binding ability of macrophages increased up to threefold. This result also suggested that changes in the concentration of cytosolic Ca^{2+} are sufficient to mediate the molecular events that lead to up-regulation of the expression of FcR. However, this finding is not always applicable to other cells. Klein et al (1990) reported that exposure of U937 cells (human monocyte-like cell line) to a calcium ionophore, ionomycin, failed to induce the expression of FcR. Therefore, the intracellular signal transduction for FcR expression may vary with the type of cell.

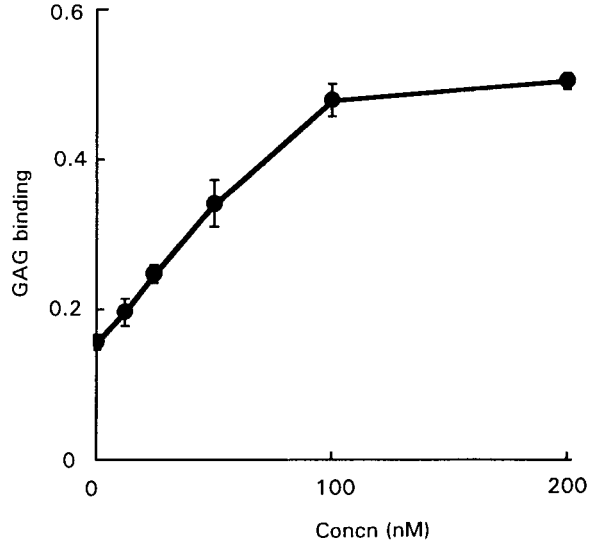

FIG. 3. Stimulatory effect of calcium ionophore, A23187, on expression of FcR of macrophages. Macrophages were cultured with varying concentrations of A23187 for 15 h, then GAG-binding activity of macrophages was measured. Each point represents the mean of four separate experiments ± s.d.

Redistribution of intracellular Ca^{2+} is frequently associated with elevation of inositol 1,4,5-trisphosphate, which is released from phosphatidylinositol bisphosphate by phospholipase C. Previously, we suggested the existence of a polysaccharide receptor on the macrophage surface which may recognize bupleuran 2IIb (Matsumoto et al 1993). It is not known whether phospholipase C is activated when bupleuran 2IIb binds to its receptor, and results in the release of inositol 1,4,5-trisphosphate. The mechanisms that transduce Ca^{2+}/calmodulin-mediated signals into the

nucleus are also unclear. Additional studies are required to define the presence of receptor and following signal transduction pathways. These studies will be important for understanding the pharmacological effect of polysaccharide and mode of action of bupleuran 2IIb.

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