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Investigation of the inhibitory effect of broussochalcone A on respiratory burst in neutrophils

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Abstract

Broussochalcone A, a prenylated chalcone isolated from *Broussonetia papyrifera* (L.) VENT. (*Moraceae*), inhibited O_2 consumption in formylmethionyl-leucyl-phenylalanine (fMLP)- and phorbol 12-myristate 13-acetate (PMA)-stimulated rat neutrophils in a concentration-dependent manner with IC_{50} values of 70.3 ± 4.9 and 63.9 ± 7.1 μM, respectively. Broussochalcone A did not affect the fMLP-induced increase of cellular inositol trisphosphate (IP_3) and $[Ca^{2+}]_i$. However, the enzyme activity of neutrophil cytosolic protein kinase C was effectively suppressed by broussochalcone A. Broussochalcone A had no effect on either [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDB) binding to neutrophil cytosolic protein kinase C or on PMA-induced membrane translocation of protein kinase C-β in neutrophils. Broussochalcone A suppressed the enzyme activity of trypsin-treated rat brain protein kinase C in a concentration-dependent manner. In PMA-activated neutrophil particulate NADPH oxidase, broussochalcone A attenuated superoxide anion radical ($O_2^{\cdot -}$) generation with an IC_{50} value of 61.8 ± 5.4 μM. These results show that the inhibitory effect of broussochalcone A on respiratory burst in neutrophils is not mediated by the reduction of phospholipase C activity, but is mediated partly by the suppression of protein kinase C activity through interference with the catalytic region and by the attenuation of $O_2^{\cdot -}$ generation from the NADPH oxidase complex.

Keywords: Broussochalcone A; Neutrophil, rat; Respiratory burst; Inositol trisphosphate; Ca^{2+} concentration, intracellular; Protein kinase C; NADPH oxidase

1. Introduction

Peripheral blood neutrophils are a major component of the body's defense against microbial invasion (Stossel, 1974). Destruction of an invading microorganism occurs as a result of a complex sequence of events. When neutrophils are stimulated by phagocytosing microorganisms or soluble agents, then the neutrophils increase their O_2 uptake from the surrounding medium and concomitantly generate large amounts of superoxide anion ($O_2^{\cdot -}$), which subsequently leads to the formation of other toxic metabolites (Badwey and Karnovsky, 1980). This non-mitochondrial O_2 consumption process is known as the respiratory burst. The concomitant generation of reactive oxygen species is believed to be important in the killing of microorganisms, and there is increasing evidence that they are also implicated in tissue damage (Tate and Repine, 1984). This is probably involved in the pathogenesis of many diseases (Halliwell and Gutteridge, 1990). A drug that would inhibit the generation of toxic oxygen radicals would terminate this tissue damage.

It has been proposed that the signal transduction mechansims in receptor-mediated neutrophil activation involves the breakdown of phosphatidylinositol 4,5-bisphosphate to give inositol trisphosphate (IP_3), which increases intracellular Ca^{2+}, and diacylglycerol, which activates protein kinase C, and that the two pathways function synergistically for $O_2^{\cdot -}$ generation (Robinson et al., 1984). Protein kinase C has been postulated to play a role in the phosphorylation of $p47^{phox}$ (Segal et al., 1985), which in turn enhances the assembly of cytosolic factors of NADPH oxidase ($p47^{phox}$ and $p67^{phox}$) to the membrane flavocytochrome b_{558} and activation of NADPH oxidase to produce $O_2^{\cdot -}$ through univalent reduction of O_2 (Segal and Abo, 1993).

Broussochalcone A (Fig. 1), a prenylated chalcone, was originally isolated from the cortex of *Broussonetia pa-*

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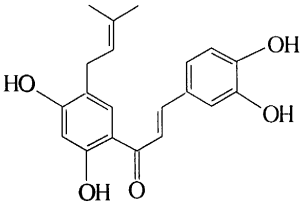

Fig. 1. Chemical structure of broussochalcone A.

pyrifera (L.) VENT. (*Moraceae*) (Matusmoto et al., 1985). The cortex of *Broussonetia papyrifera* has been used as a folk medicine for diuresis, hemostasis and the relief of edema and cough. In the preliminary study, we found that broussochalcone A inhibited the neutrophil respiratory burst in vitro. The current study examined the action mechanism of broussochalcone A by evaluating the effect of broussochalcone A on the key biochemical processes involved in the respiratory burst in neutrophils.

2. Materials and methods

2.1. Materials

Broussochalcone A was isolated and purified as previously described (Fang et al., 1994). All chemicals were purchased from Sigma (St. Louis, MO, USA), except for the following: dextran T-500 (Pharmacia Biotech, Uppsala, Sweden); 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122) (Biomol Research Laboratories, Plymouth Meeting, PA, USA); rat brain protein kinase C (Boehringer-Mannheim, Mannheim, Germany); DE-52 cellulose and Whatman GF/C filter (Whatman, Singapore); AG 1-X8 resin (formate) and electrophoresis reagents (Bio-Rad, Hercules, CA, USA); polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA); fura 2-AM (Molecular Probe, Eugene, OR, USA); [^{3}H]phorbol 12,13-dibutyrate (DuPont NEN, Boston, MA, USA); [γ-^{32}P]ATP, *myo*-[^{3}H]inositol, protein kinase C assay kit and enhanced chemiluminescence reagents (Amersham International, Amersham, UK); and anti-protein kinase C-β monoclonal antibody (Transduction Laboratories, Lexington, KY, USA). Dimethyl sulfoxide (DMSO) was the solvent used for inhibitors.

2.2. Isolation of neutrophils

Fresh blood was collected from the abdominal aorta of pentobarbital-anesthetized rats (Sprague-Dawley, 300–350 g), and the neutrophils were purified by dextran sedimentation, hypotonic lysis of erythrocytes, and centrifugation through Ficoll-Hypaque (Wang et al., 1995). Purified neutrophils containing $>95\%$ viable cells were normally resuspended in Hanks' balanced salt solution containing 4 mM $NaHCO_3$ and 10 mM HEPES, pH 7.4, (HBSS) and kept in an ice bath before use.

2.3. Respiratory burst assay

O_2 consumption by neutrophils was measured with a Clark-type oxygen electrode using a YSI oxygen monitor (Model 5300). Assays were conducted at 37°C with 6×10^6 cells which were preincubated for 5 min with stirring to permit temperature equilibration. The reaction was started by injection of the activating agent into the chamber. O_2 consumption was determined by a continuous assay as described (Ingraham et al., 1982).

2.4. Determination of inositol phosphate levels

Neutrophils (3×10^7 cells/ml) were loaded with *myo*-[^{3}H]inositol (83 Ci/mmol) at 37°C for 2 h (Wang et al., 1994). 10-s after the addition of formylmethionyl-leucyl-phenylalanine (fMLP), the reaction was stopped by adding $CHCl_3/CH_3OH$ (1:1, v/v) mixture and 2.4 M HCl. The aqueous phase was removed and neutralized with 0.4 M NaOH, and then applied to an AG 1-X8 resin column. Inositol monophosphate (IP), inositol bisphosphate (IP_2), and IP_3 were eluted sequentially by using 0.2, 0.4 and 1.0 M ammonium formate, respectively, in 0.1 M formic acid as eluents, and then counted as dpm as described in detail elsewhere (Downes and Michell, 1981).

2.5. Measurement of intracellular Ca^{2+} concentration

Neutrophils (1×10^7 cells/ml) were suspended in HEPES buffer A (124 mM NaCl, 4 mM KCl, 0.64 mM Na_2HPO_4, 0.66 mM KH_2PO_4, 15.2 mM $NaHCO_3$, 5.56 mM dextrose and 10 mM HEPES, pH 7.4), and loaded with 5 μM fura 2-AM as described previously (Wang et al., 1995). After washing, the cells were resuspended in the same buffer with 0.05% bovine serum albumin. Fluorescence was monitored with a double-wavelength fluorescence spectrophotometer (PTI, Deltascan 4000) at 510 nm with excitation at 340 and 360 nm in the ratio mode. Calibration of the excitation ratio in terms of Ca^{2+} concentration was performed as previously described (Grynkiewicz et al., 1985).

2.6. Measurement of protein kinase C activity

For the preparation of cytosolic protein kinase C, neutrophils (6×10^7 cells/ml) were disrupted in buffer A (50 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 50 mM 2-mercaptoethanol, 2 mM phenylmethylsulphonyl fluoride, 5 mM EDTA, 10 mM EGTA, 0.01% leupeptin and 10 mM benzamidine) by sonication, and centrifuged at $100\,000 \times g$. The supernatant was then applied to a DE-52 cellulose column. Protein kinase C was eluted with buffer B (50 mM Tris-HCl, pH 7.5, 50 mM 2-mercaptoethanol, 2 mM phenylmethylsulphonyl fluoride, 1 mM EDTA, 1 mM EGTA, 0.01% leupeptin and 10 mM benzamidine) containing 0.4 M NaCl (Wang et al., 1995). Neutrophil cytosolic

protein kinase C activity was assayed by measuring the incorporation of ^{32}P into peptide substrate using a protein kinase C assay kit, based on the mixed micelle method previously described (Hannun et al., 1986). Briefly, the reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5, 1 mM $CaCl_2$, 15 mM magnesium acetate, 2.5 mM dithiothreitol, 6 mM phosphatidylserine, 2 μg/ml of phorbol 12-myristate 13-acetate (PMA), 50 μM ATP (0.2 μCi [γ-^{32}P]ATP per tube), 75 μM protein kinase C substrate and protein kinase C sample. After addition of stop reagent, an aliquot of the mixture was spotted onto the phosphocellulose disc. Labeled substrate bound to binding paper was washed and counted in d.p.m. In some experiments, brain protein kinase C was partially digested with trypsin as previously described to generate the catalytic region (Inoue et al., 1977). The enzyme activity of trypsin-treated protein kinase C was determined as described above except that $CaCl_2$, phosphatidylserine and PMA were absent from the reaction mixture.

2.7. Immunoblot analysis of protein kinase C-β

Neutrophils (4×10^7 cells/ml) were stimulated with 0.2 μM PMA for 5 min at 37°C. The reaction was stopped by the addition of 4 vols. of ice-cold HBSS, and the neutrophils were then resuspended in disruption solution (0.34 M sucrose, 10 mM Tris-HCl, pH 7.0, 1 mM phenylmethylsulphonyl fluoride, 1 mM EGTA, 10 mM benzamidine, 10 μg/ml of leupeptin and antipain). After sonication, the lysate was centrifuged at $800 \times g$ for 5 min at 4°C to remove the unbroken cells, and then further centrifuged at $100\,000 \times g$ for 30 min at 4°C. The pellet (as membrane fraction) and supernatant (as cytosol fraction) were boiled in Laemmli sample buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene difluoride membrane. These membranes were incubated with 5% non-fat milk in TST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) and probed with a monoclonal antibody to protein kinase C-β (1:500 dilution in TST buffer with 0.5% non-fat milk). Detection was made using the enhanced chemiluminescence system.

2.8. [^{3}H]Phorbol 12,13-dibutyrate binding to protein kinase C

The reaction mixture contained 20 mM Tris-HCl, pH 7.2, 100 mM KCl, 50 μg/ml of phosphatidylserine, 0.5 mM $CaCl_2$, 30 nM [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDB, 20 Ci/mmol) and neutrophil cytosolic protein kinase C. For the determination of non-specific binding, 30 μM PDB was present in the reaction mixture. After addition of ice-cold 0.5% dimethyl sulfoxide (DMSO) solution to terminate the reaction, the mixture was poured onto a Whatman GF/C filter. The filter was then washed and counted in dpm as described (Tanaka et al., 1986).

2.9. Measurement of NADPH oxidase activity

Particulate NADPH oxidase was isolated as described (Wang et al., 1994) with certain modifications. Neutrophils (1×10^8 cells/ml) were incubated with 4 mM diisopropyl fluorophosphate at 4°C for 15 min, then washed twice and suspended in HBSS to 5×10^7 cells/ml. The cells were activated by 2 μM PMA in HBSS containing 1 mM NaN_3 at 37°C for 15 min. A 5-fold excess volume of ice-cold HBSS was added and the tubes were then immediately placed in a melting ice bath. After centrifugation at $500 \times g$ for 8 min, the pellets were resuspended in cold 0.34 M sucrose buffered with 10 mM Tris, pH 7.0, to 2.5×10^7 cells/ml, and sonicated in an ice-water bath for 30 s in the presence of 1 mM phenylmethylsulphonyl fluoride, 10 mM benzamidine, 10 μg/ml of leupeptin and antipain. The sonicates were centrifuged at $300 \times g$ for 8 min, and supernatants were then further centrifuged at $100\,000 \times g$ for 30 min at 4°C. The final pellets were resuspended in 0.34 M sucrose to yield a protein concentration of 2–3 mg/ml and were stored at −70°C until the time of assay. NADPH oxidase activity was measured spectrophotometrically at 28°C, based on the consumption of NADPH. The assay mixture contained 0.04% sodium deoxycholate, 12.5 μM FAD, 0.2 ml of particulate protein solution and 62.5 μM NADPH in a final volume of 1.6 ml. The oxidation of NADPH to NADP was continuously monitored at 340 nm (Wang et al., 1994). The amount of NADP produced was calculated by reference to a NADP standard curve.

2.10. Statistical analysis

Statistical analyses were performed using the Bonferroni *t*-test method after analysis of variance. Data are presented as the means ± S.E.M. A P value less than 0.05 was considered significant for all tests. Analysis of the regression line test was used to calculate IC_{50} values.

3. Results

3.1. Neutrophil O_2 consumption

Addition of 0.1 μM fMLP plus 5 μg/ml of dihydrocytochalasin B, or 10 nM PMA to the neutrophil suspensions in the presence of 1 mM NaN_3 induced non-mitochondrial O_2 consumption. Broussochalcone A inhibited O_2 consumption in rat neutrophils stimulated with fMLP/dihydrocytochalasin B as well as with PMA (Fig. 2A,B). The effects of broussochalcone A were concentration dependent with respect to both fMLP/dihydrocytochalasin B and PMA. Significant inhibition ($P < 0.01$) was observed at concentrations of broussochalcone A ≥ 50 μM for fMLP/dihydrocytochalasin B-, and ≥ 30 μM for PMA-induced responses. The IC_{50} values of broussochalcone A for the inhibition of fMLP/dihydrocytochalasin B- and

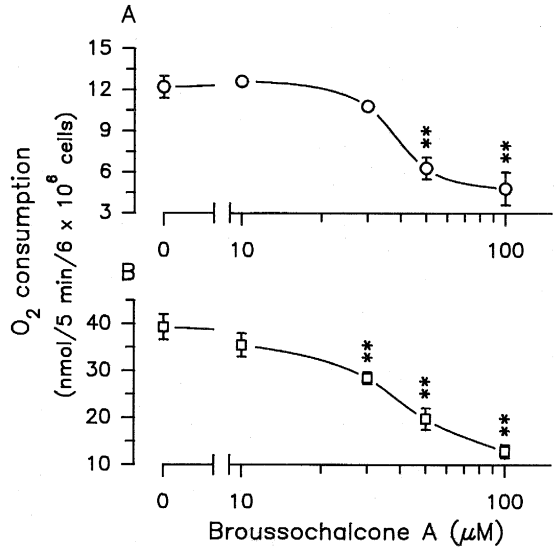

Fig. 2. Effect of broussochalcone A on O_2 consumption in neutrophils stimulated with fMLP/dihydrocytochalasin B and PMA. Neutrophils (6×10^6 cells, at 37°C) were preincubated with DMSO (as control) or various concentrations of broussochalcone A for 3 min before the addition of (A) 0.1 μM fMLP and 5 μg/ml of dihydrocytochalasin B, or (B) 10 nM PMA for 5 min. O_2 consumption was measured continuously with a Clark-type oxygen electrode as described in Section 2. The data are expressed as the means ± S.E.M. of 4–5 separate experiments. ** $P <$ 0.01 compared to the corresponding control values.

PMA-induced O_2 consumption were estimated to be 70.3 ± 4.9 μM and 63.9 ± 7.1 μM, respectively.

3.2. Inositol phosphate formation and intracellular Ca^{2+} concentration

In *myo*-[^{3}H]inositol-loaded neutrophil suspension, 0.3 μM fMLP significantly increased ($P < 0.01$) IP_3 formation. The cellular levels of IP_2 and IP_3 in response to fMLP were greatly reduced by 30 μM U73122, a phospholipase C inhibitor (Bleasdale et al., 1990). However, broussochalcone A (up to 100 μM) did not affect the responses induced by fMLP (Fig. 3). In the presence of 1 mM EDTA to remove the extracellular Ca^{2+}, 0.1 μM fMLP induced a rapid and transient elevation of $[Ca^{2+}]_i$ in fura 2-loaded neutrophils (data not shown). Cells pretreated with 1 μM U73122 did not show the fMLP-induced $[Ca^{2+}]_i$ changes. Broussochalcone A (up to 100 μM) was found to have a negligible effect on the fMLP-induced response (47.3 ± 3.8 vs. 51.6 ± 6.7 nM $[Ca^{2+}]_i$, $P > 0.05$). The low control $[Ca^{2+}]_i$ value is probably due to the relatively high concentration of fura 2-AM (5 μM) used for loading the cells, which buffers the changes induced by fMLP.

3.3. Protein kinase C activity

In the presence of $CaCl_2$, phosphatidylserine and PMA, the incorporation of ^{32}P from [γ-^{32}P]ATP into a peptide substrate was demonstrated in neutrophil cytosolic protein kinase C preparations (0.44 nmol ^{32}P/min per mg protein). Like staurosporine, a protein kinase inhibitor, broussochalcone A (10–100 μM) inhibited protein kinase C in a concentration-dependent manner (Fig. 4A,B). Significant inhibition was observed at concentrations of broussochalcone A ≥ 30 μM, and the IC_{50} value was 80.3 ± 12.3 μM.

3.4. Protein kinase C-β membrane translocation

In order to determine the subcellular distribution of protein kinase C, immunoblot analysis was carried out with specific anti-protein kinase C-β antibody. As shown in Fig. 5, protein kinase C-β was enriched in the cytosol fraction of resting cells. Upon 0.2 μM PMA treatment protein kinase C-β was translocated from cytosol to membrane. This PMA-induced response was affected by neither broussochalcone A (10–100 μM) nor staurosporine (0.1 μM). In addition, broussochalcone A (100 μM) alone did not affect the subcellular distribution of protein kinase C-β between cytosol and membrane.

3.5. Binding of [^{3}H]phorbol 12,13-dibutyrate to protein kinase C

The binding of [^{3}H]PDB to neutrophil cytosolic protein kinase C was determined by means of the rapid filtration assay. Non-specific bound [^{3}H]PDB was less than 15% of

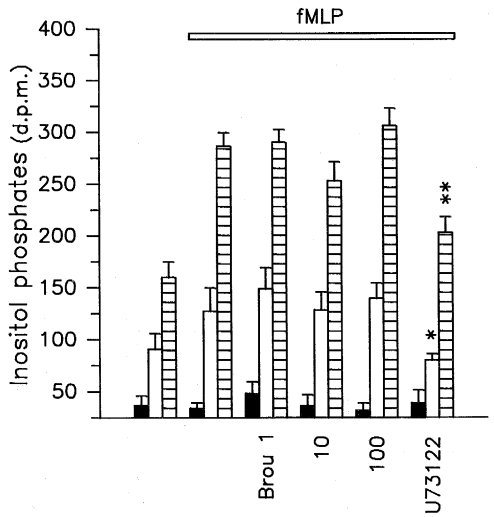

Fig. 3. Effect of broussochalcone A on fMLP-induced inositol phosphate formation in neutrophils. DMSO (as control, 2nd grouped columns), 1–100 μM broussochalcone A, or 30 μM U73122 was added to the *myo*-[^{3}H]inositol-loaded cell suspension in the presence of 10 mM LiCl at 37°C for 3 min before addition of 0.3 μM fMLP to start the reaction. After extraction and separation as described in Section 2, the levels of IP (solid column), IP_2 (open column), and IP_3 (hatched column) were counted as dpm. The resting levels of inositol phosphates are also shown (1st grouped columns). The data are expressed as means ± S.E.M. of 4–5 separate experiments. * $P < 0.05$, ** $P < 0.01$ compared to the corresponding control values.

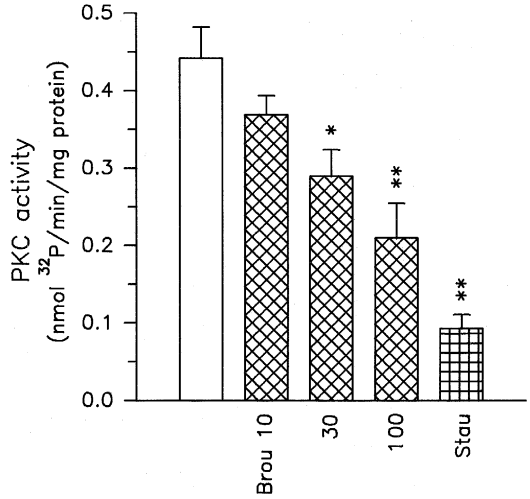

Fig. 4. Effect of broussochalcone A on protein kinase C activity. Neutrophil cytosolic protein kinase C was incubated with DMSO (as control, open column), 10–100 μM broussochalcone A, or 3 nM staurosporine for 3 min at 25°C in the presence of Ca^{2+}/phosphatidylserine, [γ-^{32}P]ATP and protein kinase C substrate before the addition of PMA to start the reaction. After termination of the reaction, phosphorylated protein was harvested in a filter, and radioactivity in the filter was counted as dpm as described in Section 2. The data are expressed as means ± S.E.M. of 3–4 separate experiments. * $P < 0.05$, ** $P < 0.01$ compared to the control value.

the total bound (270.0 ± 17.1 vs. 1873.0 ± 52.3 dpm). 1-Oleoyl-2-acetyl-*sn*-glycerol (OAG) at 10 μM greatly reduced ($P < 0.01$) the [^{3}H]PDB binding to neutrophil

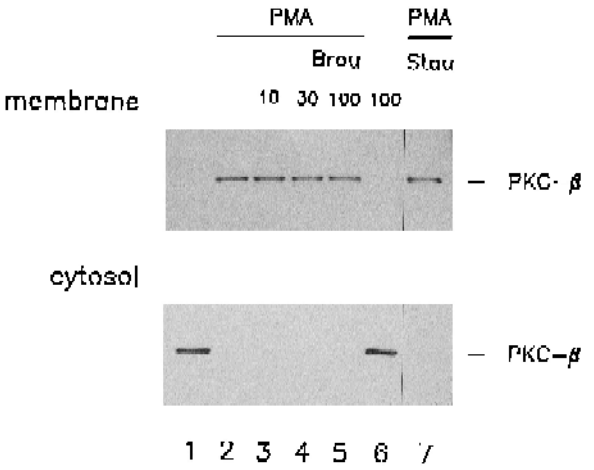

Fig. 5. Effect of broussochalcone A on protein kinase C-β membrane translocation. Neutrophils (4×10^7 cells) were preincubated with (lane 2) DMSO, (lanes 3–5) 10–100 μM broussochalcone A, or (lane 7) 0.1 μM staurosporine for 5 min at 37°C and then stimulated with 0.2 μM PMA for another 5 min. Cells may also react with (lane 1) DMSO or (lane 6) 100 μM broussochalcone A alone for a total 10 min of reaction time. After termination of the reaction, the cells were disrupted by sonication, and then centrifuged as described in Section 2. Membrane and cytosol proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Analysis was performed by immunoblotting with a monoclonal antibody to protein kinase C-β. The results shown are representative of 3 separate experiments.

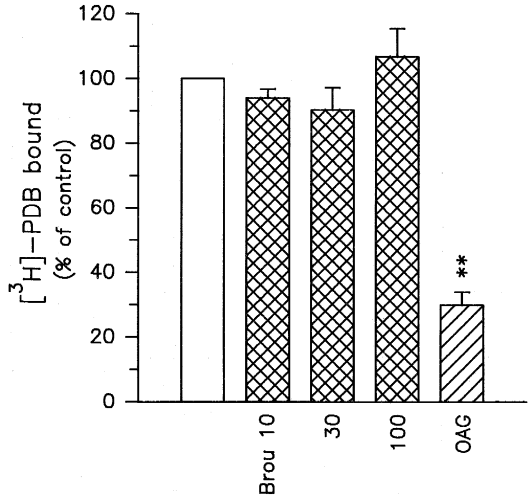

Fig. 6. Effect of broussochalcone A on [^{3}H]PDB binding to protein kinase C. Neutrophil cytosolic protein kinase C was incubated with DMSO (as control, open column), 10–100 μM broussochalcone A, or 10 μM OAG at 30°C for 3 min before the addition of [^{3}H]PDB. After termination of the reaction, protein was harvested in a filter, and radioactivity in the filter was counted in dpm as described in Section 2. The data are expressed as means ± S.E.M. of 4–5 separate experiments. ** $P < 0.01$ compared to the control value.

cytosolic protein kinase C. However, broussochalcone A (up to 100 μM) did not affect [^{3}H]PDB binding (Fig. 6).

3.6. Trypsin-treated protein kinase C activity

In a trypsin-treated rat brain protein kinase C preparation, the incorporation of ^{32}P from [γ-^{32}P]ATP into the

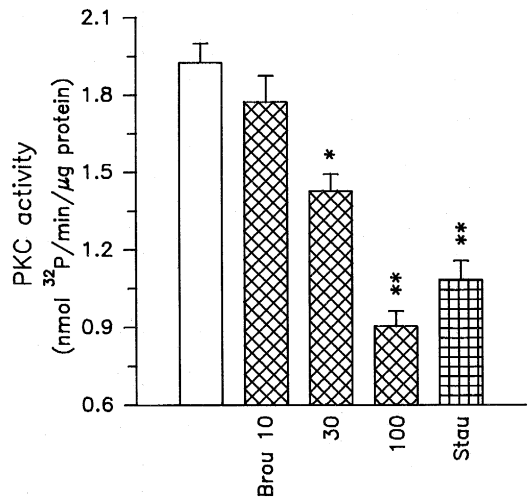

Fig. 7. Effect of broussochalcone A on trypsin-treated protein kinase C activity. Trypsin-treated rat brain protein kinase C was incubated with DMSO (as control, open column), 10–100 μM broussochalcone A, or 3 nM staurosporine for 3 min at 25°C before the addition of [γ-^{32}P]ATP and protein kinase C substrate to start the reaction. After termination of the reaction, phosphorylated protein was harvested in a filter, and radioactivity in the filter was counted as dpm as described in Section 2. The data are expressed as means ± S.E.M. of 3–4 separate experiments. * $P < 0.05$, ** $P < 0.01$ compared to the control value.

Table 1
Effect of broussochalcone A on NADPH oxidase activity

Drugs [a]	(μM)	NADPH oxidase activity [b] (nmol NADP/10 min per 5×10^6 cells eq)
Control		20.8 ± 4.9
Broussochalcone A	30	14.1 ± 2.6
	50	10.0 ± 2.8 [c]
	100	6.2 ± 1.6 [d]
Diphenylene iodonium	3	3.6 ± 1.2 [d]

[a] Drugs were preincubated with PMA-activated neutrophil particulate NADPH oxidase at 37°C for 3 min before the addition of NADPH to start the reaction.
[b] NADPH oxidase activity was measured by detecting the consumption of NADPH at 340 nm as described in Section 2. Data are expressed as means ± S.E.M. of 4–5 separate experiments.
[c] $P < 0.05$, compared to the control value.
[d] $P < 0.01$, compared to the control value.

peptide substrate was observed in the absence of Ca^{2+}/phosphatidylserine and PMA. This protein kinase C activity was effectively attenuated by broussochalcone A (≥ 30 μM) as well as by staurosporine (3 nM) (Fig. 7). The inhibition of trypsin-treated protein kinase C activity by broussochalcone A was concentration dependent with an IC_{50} value of 84.3 ± 8.6 μM.

3.7. NADPH oxidase activity

NADPH oxidase activity was examined by measuring the rate of NADPH oxidation in a PMA-activated neutrophil particulate NADPH oxidase preparation in which a functional oxidase complex had been already formed. As shown in Table 1, NADPH oxidase activity was significantly suppressed by 3 μM diphenylene iodonium (82.9 ± 2.8% inhibition, $P < 0.01$), an inhibitor of NADPH oxidase (Cross and Jones, 1986), and by broussochalcone A at concentrations ≥ 50 μM. The inhibition of NADPH oxidase activity by broussochalcone A was concentration dependent with an IC_{50} value of 61.8 ± 5.4 μM.

4. Discussion

It is well established that fMLP and PMA elicit the respiratory burst by activating the same NADPH oxidase in neutrophils, but that they utilize different transduction mechanisms and are regulated differently (McPhail and Snyderman, 1983; Segal and Abo, 1993). fMLP activates neutrophils by binding to a specific G-protein-linked receptor on the membrane (Ohta et al., 1985), whilst PMA bypasses the membrane receptor and directly activates protein kinase C (Castagna et al., 1982). The present study showed that broussochalcone A inhibited both fMLP/dihydrocytochalasin B- and PMA-induced respiratory burst in neutrophils in a concentration-dependent manner.

Activation of the membrane receptor leads to the activation of phospholipase C that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate two second messengers, IP_3 and diacylglycerol. IP_3 mobilizes Ca^{2+} from intracellular stores, leading to a transient rise in $[Ca^{2+}]_i$, whereas diacylglycerol stimulates protein kinase C (Rana and Hokin, 1990). Unlike U73122, a phospholipase C inhibitor (Bleasdale et al., 1990), broussochalcone A did not affect IP_2 and IP_3 changes in neutrophils in response to fMLP. These results lead us to suggest that broussochalcone A did not suppress phospholipase C activity. In the presence of EDTA to remove extracellular Ca^{2+}, the increase of $[Ca^{2+}]_i$ in response to fMLP results mainly from the release of Ca^{2+} from IP_3-sensitive intracellular stores. The observation that broussochalcone A had no effect on $[Ca^{2+}]_i$ in response to fMLP confirmed the suggestion.

Protein kinase C participates in the activation of NADPH oxidase, probably through the phosphorylation of $p47^{phox}$ (Kramer et al., 1988). Protein kinase C-dependent phosphorylation of $p47^{phox}$ has been reported to correlate with translocation of the cytosolic factor ($p47^{phox}$ and $p67^{phox}$) to the plasma membrane, and with the ensuing assembly of an active $O_2^{\cdot -}$-generating NADPH oxidase (Naussef et al., 1991). Like a protein kinase inhibitor, staurosporine, broussochalcone A suppressed neutrophil cytosolic protein kinase C activity in a concentration-dependent manner. The findings lead us to suggest that broussochalcone A probably reduced the respiratory burst through the inhibition of protein kinase C activity. The mammalian protein kinase C family consists of at least 12 different isoforms (Dekker and Parker, 1994), in which α, β and ζ have been identified in neutrophils (Pontremoli et al., 1990; Stasia et al., 1990). So far, we do not have evidence to indicate which of the protein kinase C isoforms was inhibited by broussochalcone A.

Protein kinase C comprises regulatory and catalytic regions (Nishizuka, 1986). The regulatory region contains C1 (diacylglycerol/phorbol ester binding site) and C2 (recognition site for acidic lipid and the Ca^{2+} binding site) domains, whereas the catalytic region contains C3 (ATP binding site) and C4 (substrate binding site) domains (Newton, 1995). Unlike OAG, broussochalcone A did not affect the [^{3}H]PDB binding to protein kinase C. These results indicate that broussochalcone A probably did not interact with the C1 domain of protein kinase C. The regulatory and catalytic regions are separated by a hinge region which is sensitive to protease. After treatment of protein kinase C with trypsin to remove the regulatory region (Lee and Bell, 1986), the remaining catalytic activity is independent of Ca^{2+}, phosphatidylserine and diacylglycerol (Inoue et al., 1977). Under these conditions, broussochalcone A, like staurosporine, a competitive inhibitor of protein kinases with respect to ATP (Tamaoki et

al., 1986), inhibited trypsin-treated protein kinase C activity. These observations suggest that the site of interaction between broussochalcone A and protein kinase C is probably at the catalytic region.

Protein kinase C activity is primarily cytosolic in unstimulated neutrophils (Wolfson et al., 1985), but becomes firmly associated with the membrane fraction after PMA treatment. Membrane translocation is mediated by diacylglycerol or phorbol ester binding to the C1 domain and phosphatidylserine/Ca^{2+} binding to the C2 domain (Newton, 1995). Isoform β is the major Ca^{2+}-dependent protein kinase C isoform and is translocated from cytosol to membrane in response to the treatment with phorbol ester (Majumdar et al., 1991). In the assay of the subcellular distribution of protein kinase C-β with the immunoblotting method, neither broussochalcone A nor staurosporine had any effect on the PMA-induced membrane associated protein kinase C-β.

Since broussochalcone A inhibited both fMLP- and PMA-induced respiratory burst in neutrophils with similar ranges of IC_{50} value, the possibility was also considered that inhibition may arise from the suppression of NADPH oxidase, the final common pathway of $O_2^{\cdot -}$ generation. In unstimulated neutrophils, NADPH oxidase is normally dormant and the membrane component (flavocytochrome b_{558}) and cytosolic factors (mainly $p47^{phox}$ and $p67^{phox}$) are not assembled. Upon stimulation, the activation of NADPH oxidase is associated with the assembly of cytosolic factors to the membrane component, thereby proceeding to the univalent reduction of O_2 in the presence of the electron donor, NADPH (Segal and Abo, 1993). In a PMA-activated neutrophil particulate NADPH oxidase preparation, a functional oxidase is already in existence, in which $p47^{phox}$ is phosphorylated and accompanied by $p67^{phox}$ to associate with membrane flavocytochrome b_{558}. The observation that broussochalcone A, like a NADPH oxidase inhibitor, diphenylene iodonium (Cross and Jones, 1986), inhibited the $O_2^{\cdot -}$ generation from the PMA-activated neutrophil particulate NADPH oxidase leads us to suggest that NADPH oxidase activity was also suppressed by broussochalcone A.

In conclusion, a natural product, broussochalcone A, attenuated the respiratory burst of neutrophils during activation. This effect may be attributable to the inhibition of protein kinase C activity by interference with the catalytic region and to the suppression of NADPH oxidase activity.

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