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Celecoxib simulates respiratory burst through pertussis toxin-sensitive G-protein, a possible signal for β_2-integrin expression on human neutrophils

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Abstract

The superoxide anion-generating effect of celecoxib (4-[5-(4-methylpheny)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide); SC58633), a selective cyclooxygenase-2 inhibitor, on human neutrophils was evaluated in this study. Celecoxib induced superoxide anion generation in a concentration-dependent manner in human neutrophils. The EC_{50} value of celecoxib on superoxide anion generation was 15.5 ± 2.5 μM. A NADPH oxidase inhibitor, diphenyliodonium (20 μM), and superoxide dismutase (150 U/ml) completely inhibited the free radical generation caused by celecoxib, indicating that the respiratory burst was activated by celecoxib. 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM;10 μM) and staurosporine (200 nM) completely inhibited the superoxide anion release caused by celecoxib, respectively. These data indicated that celecoxib increased superoxide anion release by increasing intracellular calcium and protein kinase C activation. Moreover, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-a)pyrrolo(3,4-C)-carbazole (Go-6976; 1 μM) and 3-[1-[3-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl)maleimide, methane sulfate (Ro-31-8220; 0.5 μM), specific inhibitors of conventional protein kinase C isotypes (α, β_I and β_{II}), significantly inhibited superoxide anion release caused by celecoxib. Rottlerin (5 μM), a protein kinase C δ inhibitor, did not affect the free radical generation caused by celecoxib. Celecoxib caused translocation of protein kinase C α, β_I and β_{II} from the cytosol to the cellular membrane. 2-[2-amino-3-methoxyphenyl]-4*H*-1-benzopyran-4-one (PD98059; 20 μM) and wortmannin (100 nM) did not decrease the superoxide anion generation caused by celecoxib, indicating that Mitogen-activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI3 kinase) were not involved in the respiratory burst induced by celecoxib. Pertussis toxin (2 μg/ml), a Gi-protein sensitive inhibitor, significantly inhibited superoxide anion release. Moreover, pertussis toxin significantly inhibited intracellular calcium mobilization and protein kinase C α, β_I and β_{II} translocation from the cytosol to the membrane. Celecoxib increased β_2-integrin expression on human neutrophils and this effect was inhibited by BAPTA/AM (10 μM), superoxide dismutase (150 U/ml), genistein (25 μM) and PD98059 (20 μM). This information indicated that intracellular calcium, superoxide anion, tyrosine kinase and MAP kinase are involved in β_2-integrin expression. Furthermore, BAPTA/AM, superoxide dismutase and genistein inhibited celecoxib-increased MAP kinase activity, indicating that MAP kinase is a downstream signal for β_2-integrin expression. In conclusion, celecoxib stimulates superoxide anion release from human neutrophils by activating pertussis toxin sensitive G-protein. An increase in intracellular calcium and protein kinase C α, β_I and β_{II} is involved in this process. Celecoxib also regulates β_2-integrin expression through superoxide anion release, tyrosine kinase and p42/p44 MAP kinase on human neutrophils.

Keywords: Celecoxib; Cyclooxygenase-2; Superoxide anion; Respiratory burst; Neutrophil; Protein kinase C (PKC); Ca^{2+} intracellular; Genistein

1. Introduction

Neutrophils are important phagocytic cells that provide the host with a first line of defense against acute bacterial and fungal diseases, and recurrent, severe, or unusual infections are associated with inherited defects of neutrophil function (Malech and Gallin, 1987; Bainton, 1992). Furthermore, abundant evidence links inappropriate neutrophil-mediated tissue damage to the pathogenesis of conditions such as acute respiratory distress syndrome, septicemia with multiorgan failure, ischemia-reperfusion injury and rheumatoid arthritis (Hernandez et al., 1987;

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Hogg, 1987). In response to numerous endogenous and exogenous agents neutrophils undergo chemotaxis toward sites of inflammation, both for defense against infectious agents and in various pathologic processes. At these sites, they release destructive bioactive compounds from cytoplasmic granules, perform phagocytic functions, and produce reactive oxygen species (Hiramatsu et al., 1987; Beckman et al., 1990). The release of reactive oxygen species is the so-called respiratory burst and is a crucial bactericidal mechanism. If inappropriately triggered, it can also be a major cause of pathological inflammation, lipid peroxidation and tissue damage. Chemoactic agents such as N-formylated peptides and chemokines help to orchestrate these neutrophilic functions by initiating various signaling cascades (Witko-Sarsat et al., 2000; Kitayama et al., 1997). An understanding of the mechanisms of action of neutrophil chemoattractants makes approaches for the selective pharmacological manipulation of neutrophil possible. Such manipulations have the potential to enhance host defense activities and to ameliorate conditions, such as inflammatory bowel disease and various forms of arthritis, in which these neutrophil functions are thought to play pathogenic roles (Parkos et al., 1994).

Rheumatoid arthritis is one of the commonest autoimmune diseases. It is a chronic progressive, systemic inflammatory disorder affecting the synovial joints and typically causes symmetrical arthritis. Neutrophils are also involved in the progression of this autoimmune disease. After infiltration of the synovium and ingestion of immune complexes, neutrophils can release lysosomal enzymes and destructive oxygen free radicals that damage cartilage and supportive joint structures. For this reason, long-term therapy for pain relief is required. Currently prescribed non-steroidal anti-inflammatory drugs (NSAIDs) provide symptomatic efficacy, but are frequently associated with gastrointestinal toxicity, such as dyspepsia and ulceration. This gastrointestinal toxicity is caused by conventional NSAIDs which inhibit cyclooxygenase-1 and cyclooxygenase-2 to the same extent. However, a selective cyclooxygenase-2 inhibitor, celecoxib (4-[5-(4-methylpheny)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl] benzene-sulfonamide), consistently demonstrates an efficacy comparable to that of conventional NSAIDs in patients with rheumatoid arthritis, but has a significantly reduced propensity to cause gastrointestinal toxicity (Lanas, 2002; Sundy, 2001).

Celecoxib may also have therapeutic potential for the treatment of colorectal cancer. The precise mechanism for inhibition of tumor growth is under evaluation. In recent studies, celecoxib was reported to induce apoptosis in prostate cancer cells by interfering with multiple signaling targets, including Akt, extracellular signal-regulation kinase-2 (ERK2), and endoplasmic reticulum Ca^{2+}-ATPase (Hsu et al., 2000). Disruption of these signaling pathways leads rapidly to apoptosis, an apoptotic mechanism distinctly different from that of conventional anticancer agents. Nevertheless, this rapid induction of apoptosis was unique to celecoxib, and the ability of other cyclooxygenase-2 inhibitors, including rofecoxib, NS398, and DuP697, to induce apoptosis was much lower than that of celecoxib (Thun et al., 2002). This observation underscores differences in the mechanisms by which these cyclooxygenase-2 inhibitors mediate apoptosis in prostate cancer cells.

In this study, celecoxib potently induced the respiratory burst in human neutrophils and this effect was exerted through activation of a pertussis toxin-sensitive G-protein, mediated by an increase in intracellular calcium and protein kinase C. Furthermore, celecoxib-induced superoxide anion regulated β_2-integrin expression on human neutrophils.

2. Materials and methods

2.1. Materials

Celecoxib was discovered by Penning et al. (1997) and is under clinical evaluation. Celecoxib was provided by Lin Yin-Chou MD and was dissolved in dimethyl sulfoxide (DMSO). 2-[2-amino-3-methoxyphenyl]-4*H*-1-Benzopyran-4-one (PD98059), wortmannin, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), Fura-2 acetoxymethylester (Fura-2/AM), pertussis toxin, rottlerin, 1,2-bis(2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA/AM), phorbol 12-myristate 13-acetate (PMA), diphenyliodonium, superoxide dismutase, staurosporine, genestin and Hank's buffered saline (HBSS) were purchased from Sigma (St. Louis, MO, USA). Hydroethidium was purchased from Molecular Probe (Eugene, OR, USA). 12-(2-cyanoethyl)-6, 7, 12, 13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2, 3-a)pyrrolo(3, 4-C)-carbazole (Go-6976) was purchased from Bio-mol (Plymouth Meeting, PA, USA). 3-[1-[3-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl)maleimide, methane sulfate (Ro-31-8220) was purchased from Calbiochem (La Jolla, CA, USA). Ficoll, horseradish-peroxidase-coupled antibody and the enhanced chemiluminescence detection agent were purchased from Amersham Pharmacia (Piscatway, NJ, USA). CD18 (β_2-integrin) fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal antibody was purchased from BD PharmMingen Technical (San Diego, CA, USA). Phospho-p44/42 mitogen-activated protein (MAP) kinase antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Conventional protein kinase C α (c20): sc-208 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Conventional protein kinase C β_I (sc-209), β_{II} (sc-210) and γ (sc-211) antibodies were gifts from Dr. Yung Cheun Mao.

2.2. Preparation of neutrophils

Venous blood samples were collected with syringes containing heparin (final concentration 20 U/ml) from healthy

volunteers of both sexes between 20 and 40 years old. Neutrophils were isolated by the Ficoll gradient centrifugation method, followed by hypotonic lysis of contamination erythrocytes (Tomita et al., 1984). Briefly, blood samples were mixed with an equal volume of 3% dextran solution in a 50-ml centrifuge tube and incubated in an upright position for 20 min at room temperature, to allow sedimentation of erythrocytes. The upper, leukocyte-rich layer was then collected and subjected to centrifugation at 250 × *g* for 15 min at 4 °C. After centrifugation, the pellet was re-suspended immediately in a volume of phosphate-buffered saline (PBS) equal to the starting volume of blood. The cell suspension was then apportioned, 25 ml per tube, into 50-ml centrifuge tubes, followed by layering of 10 ml of 1.077 g/ml Ficoll solution beneath the cell suspension, using a pipette. After centrifugation at 400 × *g* for 40 min at 20 °C without braking, the upper (PBS) and lower (Ficoll) layers were carefully removed, leaving the granulocyte/erythrocyte pellet. To remove residual erythrocytes, the pellet was re-suspended in 20 ml cold 0.2% NaCl for 30 s, followed by addition of 20 ml cold 1.6% NaCl to restore tonicity. The remaining neutrophils were then pelletted, washed twice with ice-cold PBS and re-suspended in an adequate volume of ice-cold Hank's buffered saline (HBSS) until further manipulation. The preparation contained more than 95% neutrophils, as estimated by counting 200 cells under a microscope after Giemsa staining.

2.3. Superoxide anion measurement

Intracellular production of superoxide anion was analyzed on a flow cytometer according to Carter et al. (1994)

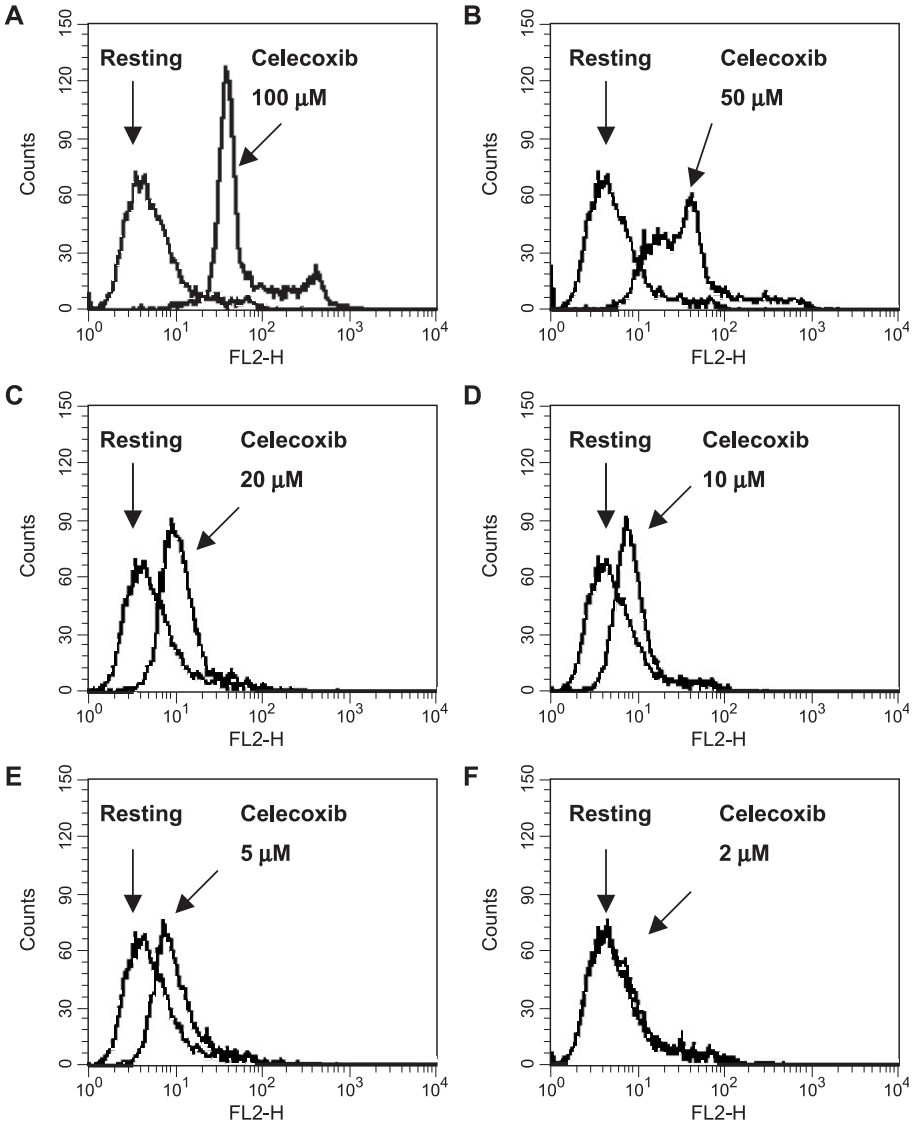


Fig. 1. Celecoxib-induced superoxide anion release from human neutrophils. (A–F) Results for flow cytometric analysis of superoxide anion release caused by various concentrations of celecoxib (A: 100 μM; B: 50 μM; C: 20 μM; D: 10 μM; E: 5 μM; F: 2 μM). Neutrophils were stained with hydroethidium (10 μM) for 15 min, followed by incubation with celecoxib for 10 min and were then monitored with FACScan.

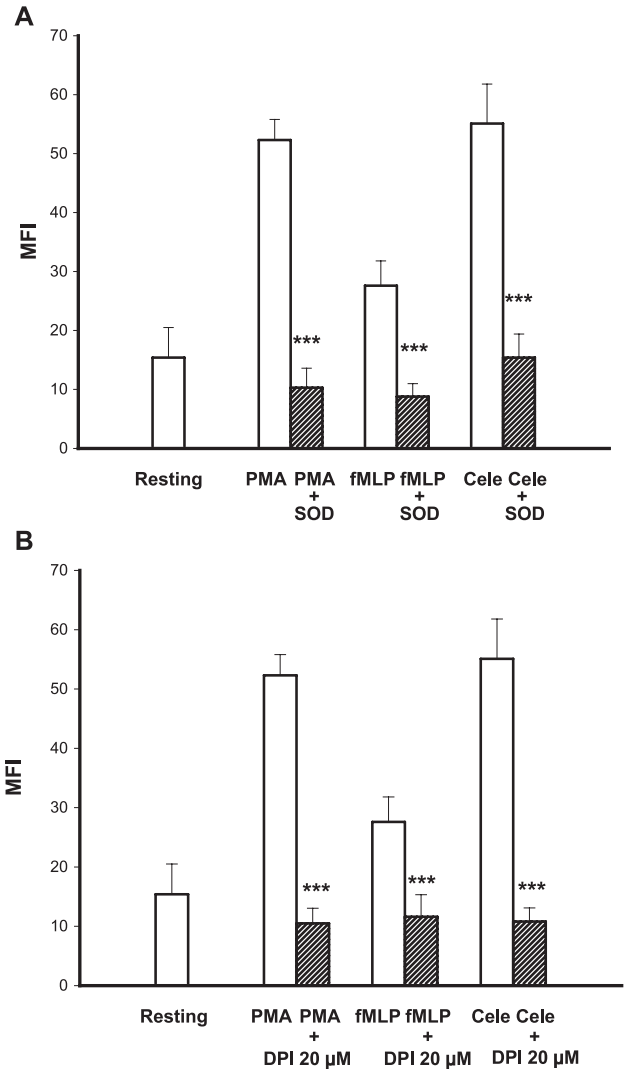


Fig. 2. Celecoxib (cele)-induced superoxide anion release was decreased by superoxide dismutase (SOD) and by the NADPHoxidase inhibitor, diphenyliodonium (DPI). Results are from flow cytometric analysis and data are shown as mean fluorescence intensity (MFI). (A) shows PMA (100 ng/ml; MFI: 52.3 ± 3.5), fMLP (1 μM; MFI: 27.6 ± 4.2) and celecoxib (100 μM; MFI: 55.5 ± 6.7) increased superoxide anion release from human neutrophils. SOD (150 U/ml) completely inhibited superoxide anion release caused by PMA (MFI: 10.3 ± 3.3), fMLP (MFI: 8.8 ± 2.2) and celecoxib (MFI: 15.4 ± 4.6). (B) shows diphenyliodonium (DPI; 20 μM) inhibited superoxide anion release from neutrophils caused by PMA (MFI: 10.5 ± 2.6), fMLP (MFI: 11.6 ± 3.7) or celecoxib (MFI: 10.8 ± 2.3). $n = 6$; ***: $p < 0.001$ compared with respective control. MFI value of resting neutrophils was 15.4 ± 5.1. Neither SOD nor DPI alone affected basal the MFI.

Briefly, neutrophils were incubated at 37 °C for 15 min with 10 μM of hydroethidium (Molecular Probe). Hydroethidium can be directly oxidized by superoxide anion to ethidium bromide, which fluoresces after intercalating with nucleic acids. After labeling, cells were treated with various concentrations of celecoxib, fMLP (1 μM) and PMA (100 ng/ml). Pertussis toxin (2 μg/ml) was pretreated 40 min before celecoxib or fMLP. Go-6976 (1 μM; protein kinase C α and β_I inhibitor), Ro-31-8220 (0.5 μM; protein kinase C β_{II} inhibitor), staurosporine (200 nM; nonselective protein kinase C inhibitor), BAPTA/AM (10 μM), superoxide dismutase (150 U/ml; superoxide anion scavenger), rottlerin (5 μM; protein kinase C δ inhibitor), PD98059 (20 μM; MAP kinase inhibitor) and wortmannin (100 nM; phosphatidylinositol 3-kinase (PI3K) inhibitor) were added 10 min before celecoxib. Production of superoxide anion was monitored every 10 min on FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) by measuring emission at 590 nm for ethidium bromide. In total 10,000 cells were collected for measurement of fluorescence intensity for each sample. Data are expressed as mean values of fluorescence intensity (MFI) for each sample as described above.

2.4. Intracellular calcium measurement

The method of Pollock and Rink (1986) was modified. Briefly, neutrophils (1×10^6 cells/ml) were incubated with fura-2/AM (2 μM) at 37 °C for 30 min and centrifuged at $200 \times g$. The resultant pellet was washed with HBSS. After centrifugation, neutrophils were re-suspended in HBSS containing calcium (1 mM). Fluorescence (excitation 340 and 380 mm; emission 500 nm) was measured with a Hitachi fluorescence spectrophotometer (model F4500; Tokyo, Japan) at 37 °C. At the end of the experiment, the cells were treated with Triton X-100 (0.1%) followed by

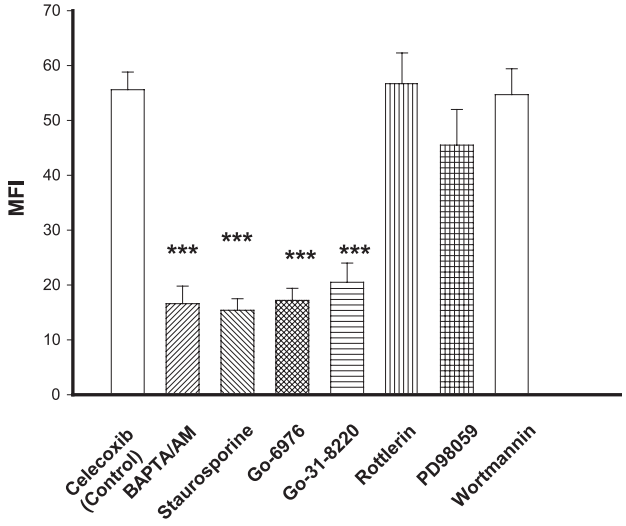


Fig. 3. Role of PKC, intracellular calcium, MAP kinase and PI3 kinase on celecoxib-induced superoxide anion release. Neutrophils were pretreated with DMSO (0.05%; control), BAPTA/AM (10 μM), staursporine (200 nM), Go-6976 (1 μM), Ro-31-8220 (0.5 μM), rottlerin (5 μM), PD98059 (20 μM) or wortmannin (100 nM) for 10 min and then celecoxib (100 μM) was added. Superoxide anion release was monitored with a flow cytometer for 10 min after celecoxib addition. Data are shown as the mean fluorescence intensity (MFI). MFI was 55.6 ± 3.2 (celecoxib; control), 17.2 ± 2.2 (Go-6976), 20.5 ± 3.5 (Ro-31-8220), 15.4 ± 2.1 (staursporine), 16.6 ± 3.2 (BAPTA/AM), 56.7 ± 5.6 (rottlerin), 45.5 ± 6.5 (PD98059) or 54.7 ± 4.7 (wortmannin), respectively. ***: $p < 0.001$ compared with celecoxib alone (control). $n = 6$.

addition of EGTA (10 mM) to obtain the maximal and minimal fluorescence, respectively. Intracellular calcium was calculated as described for fura-2, using the calcium-dye dissociation constant 224 nM.

2.5. Preparation of subcellular fractions

The method of Majumdar et al. (1991) was modified. Briefly, neutrophils (5×10^7 cells ml^{-1}) were allowed to warm for 5 min at 37 °C. Cells were stimulated by the addition of warmed HBSS or celecoxib (100 μM). The stimulation was stopped at predetermined times by the addition of 10 volumes of ice-cold HBSS followed by centrifugation ($250 \times g$, 10 min, 4 °C). Neutrophils were re-suspended to a concentration of 5×10^7 cells ml^{-1} in the appropriate ice-cold extraction buffer and sonicated for 5–10 s at 4 °C (whole sonicate fraction), followed by centrifugation at $100{,}000 \times g$ for 1 h at 4 °C. The supernatant (cytosol fraction) was collected and the pellet re-suspended by sonication (5 s) at 4 °C to the original volume in extraction buffer (particulate fraction). For the translocation studies, 0.2% Triton X-100 was added to the pellet before sonication. Fractions were stored at -80 °C and assayed within 48 h.

For analysis of ERK activity, neutrophils (2×10^6 cells/ml) were incubated with superoxide dismutase (150 U/ml), genistein (25 μM), PD98059 (20 μM) and BAPTA/AM (10 μM) for 10 min at 37 °C and then stimulated with 1 μM fMLP, 100 ng/ml PMA or 100 μM celecoxib. Stimulation was terminated by placing the cells on ice, subjecting them to immediate centrifugation, and re-suspending the cell pellets in 1 × Laemmli sample buffer. After boiling for 10 min, the proteins were stored in −70 °C for immunoblotting assay.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoesis (SDS-PAGE) and immunoblotting

The sample was electrophoresed in 8–10% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose (Laemmli, 1970, Towbin et al., 1979). Blots were stained with Ponceau-S (0.2% Ponceau-S 3% trichloroacetic

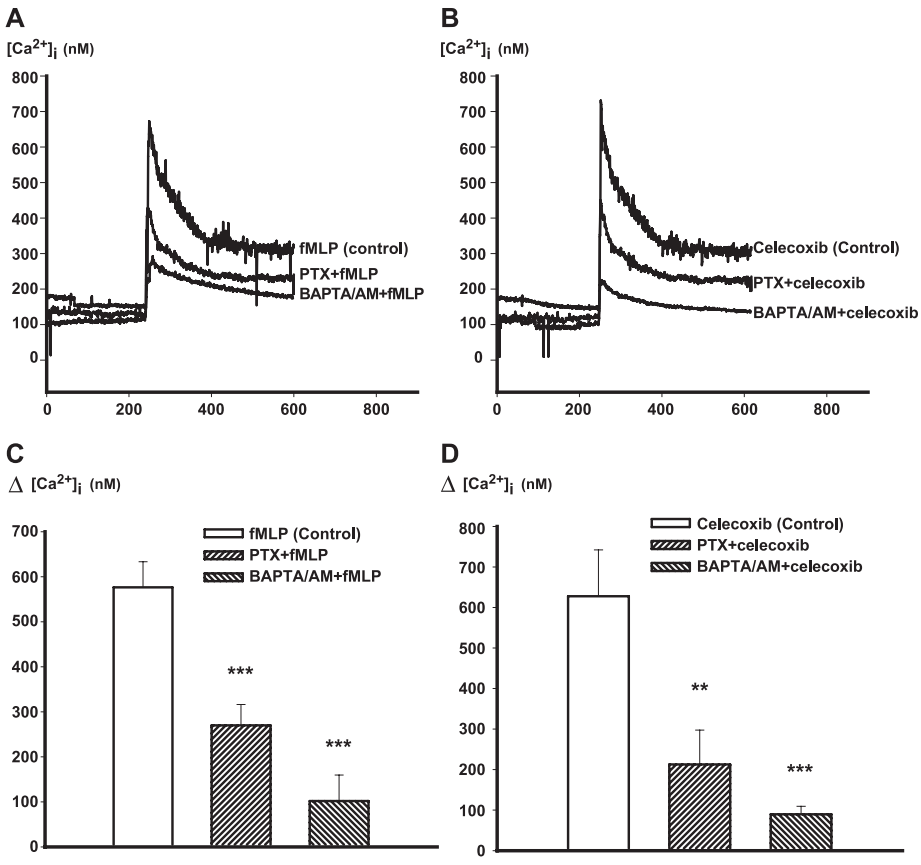


Fig. 4. Role of pertussis toxin (PTX) and BAPTA/AM on celecoxib- and fMLP-induced intracellular calcium mobilization. Neutrophils were stained with 2 μM fura-2/AM for 30 min. Intracellular calcium was monitored with a fluorescence spectrophotometer. (A) A typical pattern of fMLP (1 μM)-induced intracellular calcium mobilization. fMLP-induced intracellular calcium mobilization was inhibited by pertussis toxin (2 μg/ml) and BAPTA/AM (10 μM). (B) shows a typical pattern of celecoxib (100 μM)-induced intracellular calcium mobilization. Celecoxib-induced intracellular calcium mobilization was inhibited by pertussis toxin (2 μg/ml) and BAPTA/AM (10 μM). (C) fMLP increased the intracellular calcium concentration. Data are shown as peak calcium concentration minus the basal calcium concentration from (A). (D) Celecoxib increased the intracellular calcium concentration. Data are shown as peak calcium concentration minus the basal calcium concentration from (B). **: $p < 0.01$, ***: $p < 0.001$ compared with control.

acid, and 3% sulfosalicylic acid) to visualize Mr markers and were destained with deionized water followed by Tris-buffered saline containing 0.1% Tween 20 (TBST; 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween-20). Blots were blocked for 1 h in 5% nonfat milk in TBST. Antibodies were diluted in PBS (pH 7.3) containing 3% bovine serum albumin and 0.02% sodium azide. Blots were incubated with the appropriate antibody (protein kinase C α, β_I, β_{II} and γ antibodies for protein kinase C translocation; Phospho-p44/42 MAP kinase antibody for MAP kinase activity) for 2 h at 25 °C, followed by thorough washing (three times, 10 min each time) with TBST. Next, blots were incubated for 1 h with an appropriate horseradish peroxidase-conjugated secondary antibody (1/5000) in 5% nonfat milk in TBST, washed thoroughly, and visualized by enhanced chemiluminescence. Autoradiographs were analyzed and quantified by densitometry.

2.7. Measurement of β_2-integrin up-regulation by flow cytometry

Expression of β_2-integrin was analyzed as described by Endemann et al. (1996) with some modification. Neutrophils (1×10^6 cells/ml) were incubated with superoxide dismutase (150 U/ml), genistein (25 μM) or BAPTA/AM (10 μM) 10 min before the addition of celecoxib (100 μM) in the presence of 20 μg/ml of FITC-conjugated anti-β_2-integrin antibody or a nonspecific mouse immunoglobulin G (IgG) as a negative control. Cells were fixed with 1% paraformaldehyde 30 min after incubation and analyzed with a flow cytometer.

2.8. Statistical analysis

Results are expressed as the means ± S.E.M. for the indicated number of separate experiments. Statistical significance between drug-treated and untreated groups were evaluated by Student's *t*-test and *P* values less than 0.05 were considered significant.

3. Results

3.1. Celecoxib stimulated the respiratory burst and superoxide anion release from human neutrophils

Superoxide anion oxidized hydroethidium to ethidium bromide, as monitored by the mean fluorescence of intensity (MFI). A typical pattern of the increase in superoxide anion generation induced by celecoxib in a concentration-dependent manner is shown in Fig. 1(A–F). Celecoxib 100 μM had a maximum effect (MFI: 49.4 ± 11.9, $n = 6$; Fig. 1A). The EC_{50} value of celecoxib on superoxide anion generation was 15.5 ± 2.5 μM ($n = 6$) The superoxide anion generated by celecoxib (100 μM) was scavenged by superoxide dismutase (150 U/ml; MFI: 15.4 ± 4.0, $P < 0.001$ compared with celecoxib alone; Fig. 2A) and diphenyliodonium (20 μM; MFI: 10.8 ± 2.3, $P < 0.001$ compared with celecoxib alone; Fig. 2B). PMA (100 ng/ml) and fMLP (1 μM) both significantly increased superoxide anion production in neutrophils (MFI: PMA: 52.3 ± 3.5, fMLP: 27.6 ± 4.2, $n = 6$). These effects were inhibited by superoxide dismutase (150 U/ml; MFI for PMA: 10.3 ± 3.3, fMLP: 8.8 ± 2.2, $n = 6$)

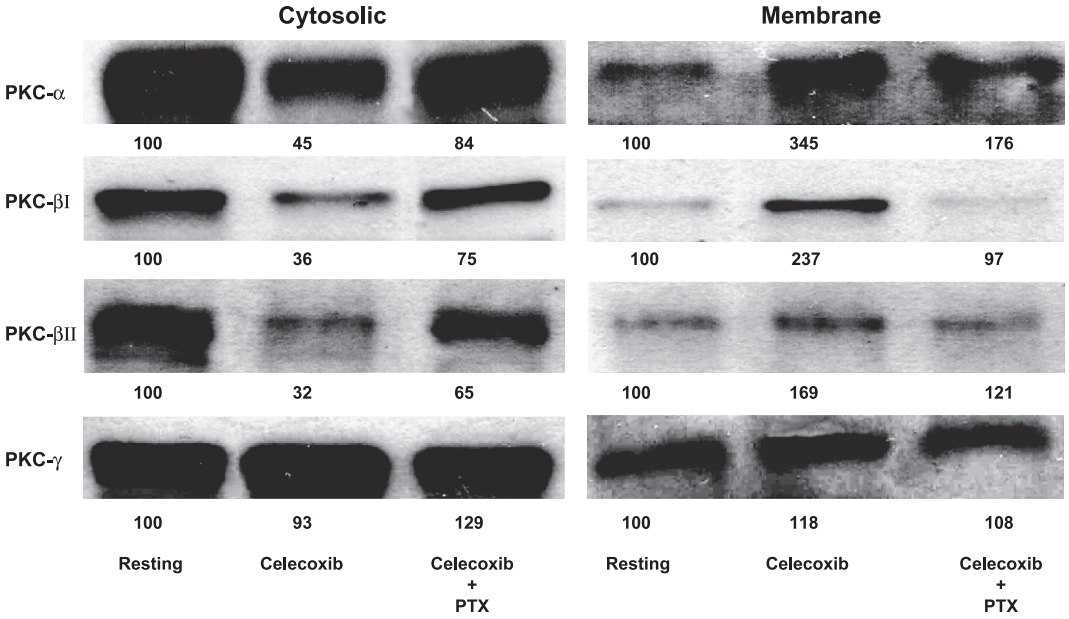


Fig. 5. Typical pattern of translocation of PKC α, β_I, β_{II} and γ from the cytosol to the membrane in human neutrophils induced by celecoxib (100 μM). Neutrophils were treated with DMSO (0.05 %) or PTX (2 μg/ml) 40 min before celecoxib was added. PKC α, β_I, β_{II} and γ protein levels were determined 15 min after celecoxib was added. The data below each trace indicate the percentage of immunoreactivity as compared with the resting level (100). The results are representative of three experiments.

and diphenyliodonium (20 μM; MFI for PMA: 10.5 ± 2.6, fMLP: 11.6 ± 3.7, $n = 6$), respectively (Fig. 2A,B). The resting level of MFI was 15.2 ± 5.2 ($n = 6$).

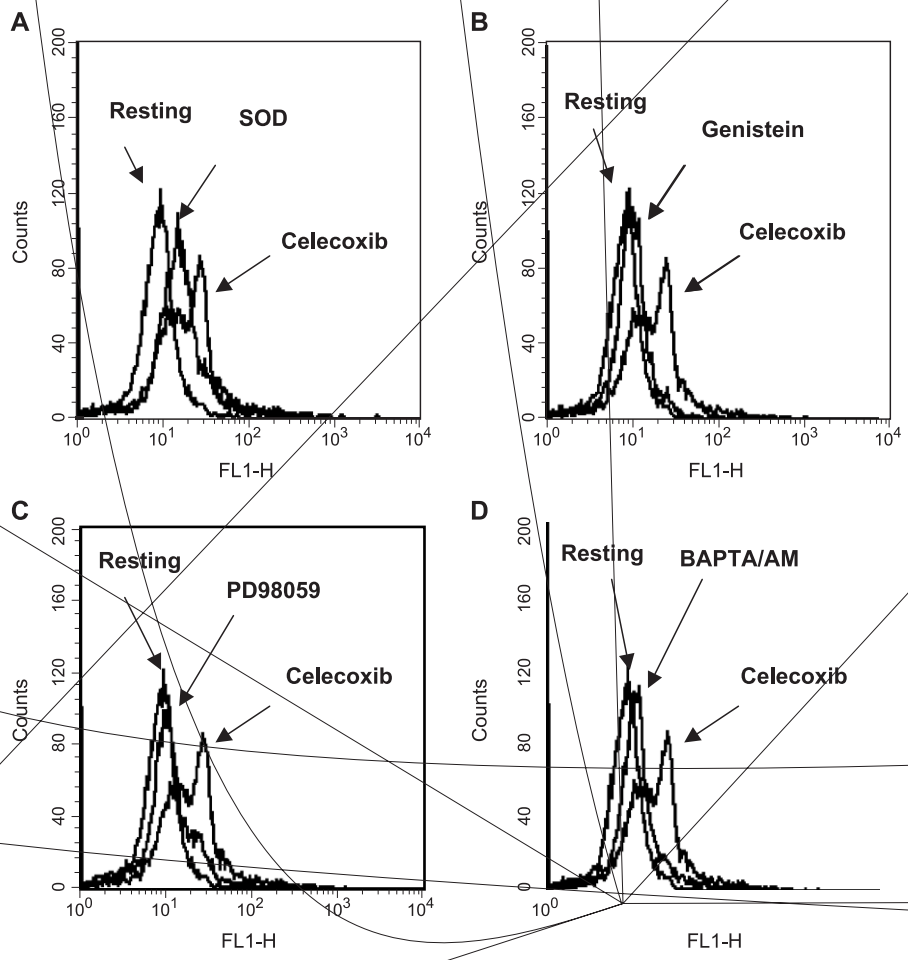
3.2. Effect of intracellular Ca^{2+}, protein kinase C, MAP kinase and PI3-kinase on celecoxib-induced superoxide anion release

Celecoxib (100 μM) significantly increased superoxide anion release from human neutrophils (Fig. 3; MFI: 55.6 ± 3.2). BAPTA/AM (10 μM), an intracellular calcium chelator, completely inhibited the superoxide anion generation induced by celecoxib (Fig. 3; MFI: 16.6 ± 3.2; $P < 0.001$ compared with celecoxib alone). Staurosporine (200 nM) inhibited superoxide anion release induced by celecoxib in human neutrophils (Fig. 3; MFI: 15.4 ± 2.1; $P < 0.00.1$ compared with celecoxib alone). Furthermore, a calcium-dependent protein kinase C inhibitor, Go-6976 (1 μM; MFI: 17.2 ± 2.2) and Ro-31-8220 (0.5 μM; MFI: 20.5 ± 3.5) inhibited celecoxib-induced free radical generation (Fig. 3). Rottlerin (5 μM), PD98059 (20 μM) and wortmannin (100 nM) did not affect the superoxide anion generation caused by celecoxib (Fig. 3).

3.3. Pertussis toxin-sensitive G-protein on celecoxib-induced intracellular calcium mobilization, protein kinase C translocation and superoxide anion release

Celecoxib increased intracellular calcium mobilization in a concentration-dependent manner. A typical pattern of intracellular calcium mobilization caused by fMLP (1 μM) and celecoxib (100 μM) is shown in Fig. 4A,B, respectively. BAPTA/AM (10 μM) significantly inhibited the intracellular calcium mobilization caused by fMLP or celecoxib (Fig. 4A,B).

The basal intracellular calcium concentration was 105.4 ± 7.2 nM. In the presence of pertussis toxin (2 μg/ml), the basal intracellular calcium concentration was 162.4 ± 11.2 nM. fMLP (1 μM) or celecoxib (100 μM) increased intracellular calcium to 716.4 ± 56.8 or 827.7 ± 114.2 nM, respectively (Fig. 4A,B). Pertussis toxin (2 μg ml^{-1}) significantly inhibited fMLP (429.2 ± 46.5 nM;



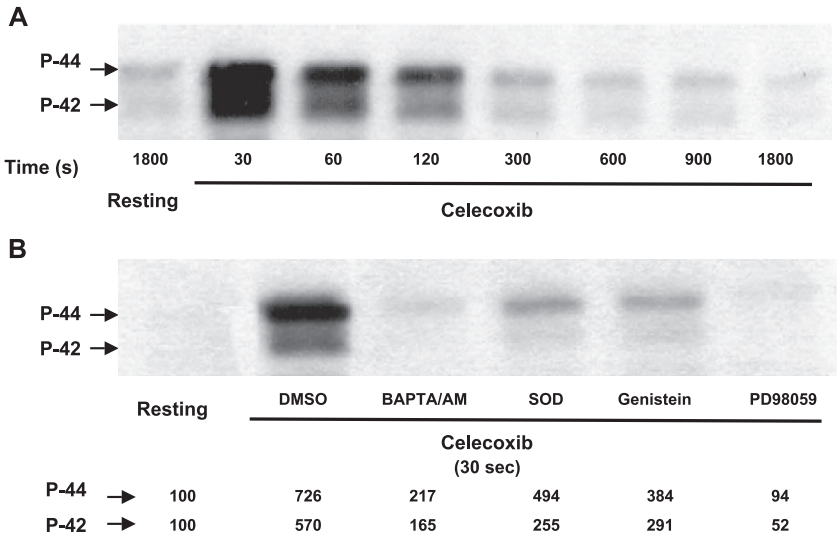


Fig. 7. Western blotting to detect phosphor-p42/p44 MAP kinase protein caused by celecoxib at different times (A). Cells were treated with celecoxib (100 μM) for different times as indicated (30–1800 s), and then phosphor-p42/p44 MAP kinase protein levels were determined. (B) Effects of BAPTA/AM, SOD, genistein and PD98059 on celecoxib-induced p42/p44 MAP kinase activity. Cells were treated with BAPTA/AM (10 μM), SOD (150 U/ml), genistein (25 μM) or PD98059 (20 μM) for 10 min before celecoxib (100 μM) was added and then phosphor-p42/p44 MAP kinase protein levels were determined at 30 s. The data below each trace indicate the percentage of immunoreactivity as compared with the resting level (100). The results are representative of three experiments.

$P<0.01$ compared with fMLP alone)- or celecoxib (462.6 ± 84.7 nM; $P<0.05$ compared with celecoxib alone)-induced intracellular calcium mobilization (Fig. 4A,B). Fig. 4C,D shows the peak calcium concentration after subtraction of the basal calcium concentration. The fMLP- and celecoxib-increased calcium concentration was 576.4 ± 56.8 and 627.7 ± 114.2 nM, respectively. In the presence of pertussis toxin, the increase in calcium induced by fMLP or celecoxib was 269.7 ± 46.5 ($P<0.01$ compared with fMLP alone) or 212.6 ± 84.7 ($P<0.001$ compared with celecoxib alone) nM, respectively. The fMLP- or celecoxib-induced increase in intracellular calcium concentration was 101.7 ± 57.8 or 89.7 ± 19.7 nM in the presence of BAPTA/AM.

Celecoxib (100 μM) elicited the translocation of protein kinase C α, β_I and β_{II} from the cytosol to the cell membrane (Fig. 5). Translocation of these protein kinase C isoforms induced by celecoxib was affected by pretreatment with pertussis toxin (2 μg/ml) for 40 min. However, celecoxib did not induce the translocation of protein kinase C γ from the cytosol to the cell membrane.

Celecoxib (100 μM) increased the concentration of superoxide anion and this effect was significantly inhibited by pertussis toxin (2 μg/ml) (MFI: 15.6 ± 1.1; $P<0.01$ compared with celecoxib alone, MFI: 29.6 ± 4.1). The basal MFI was 10.1 ± 0.2 ($n=6$).

3.4. Signals regulating β_2-integrin expression caused by celecoxib

A typical pattern of the celecoxib (100 μM)-induced increase in β_2-integrin expression is shown in Fig. 6. β_2-integerin expression induced by celecoxib was significantly inhibited by superoxide dismutase (150 U/ml; Fig. 6A), genistein (25 μM; Fig. 6B), PD98059 (20 μM; Fig. 6C) and BAPTA/AM (10 μM) (Fig. 6D). The resting level of MFI was 11.4 ± 0.8 and increased to 21.5 ± 1.9 in the presence of celecoxib (100 μM) (Fig. 6). The MFI values for celecoxib in the presence of superoxide dismutase, genistein, PD98059 and BAPTA/AM were 16.0 ± 1.0 ($n=6$; $P<0.05$ compared with celecoxib alone), 14.9 ± 0.8 ($n=6$; $P<0.01$ compared with celecoxib alone), 15.7 ± 1.1 ($n=6$; $P<0.01$ compared with celecoxib alone) and 14.2 ± 0.6 ($n=6$; $P<0.01$ compared with celecoxib alone), respectively.

To determine the interaction between superoxide anion, tyrosine kinase and MAP kinase on celecoxib up-regulated β_2-integrin expression, MAP kinase activity was evaluated in the presence of different inhibitors. p-42/p44 MAP kinase activity was significantly increased and reached a maximum at 30 s after being stimulated with celecoxib (100 μM) and declined after 5 min (Fig. 7A). Superoxide dismutase (150 U/ml), genistein (25 μM), PD98059 (20 μM) and BAPTA/AM (10 μM) significantly inhibited p-42/p44 MAP kinase activity caused by celecoxib (Fig. 7B).

4. Discussion

Celecoxib belongs to the new generation of NSAIDs. Although celecoxib reduces many inflammatory responses (Wilgus et al., 2002; Chen et al., 2002), several unexpected adverse effects, including those in patients with coronary artery disease and with renal dysfunction (Ring 2003; Ozturk et al., 2002) have been reported. Therefore, care should be exercised in administering specific cyclooxyge-

nase-2 inhibitors to patients with pre-existing cardiac or renal disease. In this study, celecoxib activated the defense mechanisms of neutrophils by triggering the respiratory burst. Therefore, the mechanisms by which neutrophils were activated by celecoxib were evaluated. Possible toxicity was excluded by using methylthiazoletetrazolium (MTT) and lactate dehydrogenase (LDH) assays (data not shown). A cyclooxygenase-2 inhibitory effect was also excluded because indomethacin did not increase free radical generation in human neutrophils (data not shown). Although several studies showed that MAP kinase and PI3-kinase were involved in the regulation of neutrophil function (Zu et al., 1998; Suzuki et al, 2001; Cadwallader et al., 2002), the celecoxib induction of free radical generation was not affected by PD98059 or wortmannin, respectively.

Staurosporine completely inhibited the free radical generation caused by celecoxib in human neutrophils. This indicates that celecoxib produces free radicals through the activation of protein kinase C. One approach to examine the potential for protein kinase C to regulate the response to phagocytic stimuli is to determine the changes in the subcellular location of protein kinase C after challenging cells with phagocytic stimuli. Protein kinase C translocation is associated with activation of the enzyme (Hug and Sarre, 1993) and has been observed upon stimulation of neutrophils with soluble stimuli (Wolfson et al., 1985; Christiansen and Borregaard, 1989). Deli et al. (1987) have provided immunocytochemical evidence for the translocation of protein kinase C from resting neutrophil cytosol to plasma and phagosomal membranes upon phagocytosis of opsonized particles. Eleven isoforms of protein kinase C have been identified and are classified into three groups, conventional protein kinase C, novel protein kinase C, and atypical protein kinase C, based on their cofactor requirements and their structure. Conventional protein kinase C (-α, -$\beta_{I/II}$ and -γ) isoforms are calcium dependent and require phosphatidylserine and diacylglycerol for activity. The novel protein kinase C (-δ, -ε ,-η, -θ and μ) isoforms can be activated by diacylglycerol and phosphatidylserine but not by calcium. Stimulation of the atypical protein kinase C (-ζ and -λ) isoforms is independent of calcium or diacyglycerol (Nishizuka, 1995). Like many cell types, human neutrophils express protein kinase C isoforms from each of the three classes (α, β_I, β_{II}, δ, and ζ), all of which are predominantly cytosolic in resting cells (Majumdar et al., 1991, Sergeant and McPhail, 1997). Determining the subcellular redistribution of these protein kinase C isoforms following celecoxib challenge should help us gain a better insight into the potential roles of celecoxib in free radical generation by neutrophils. The results of the present study showed that celecoxib induced the translocation of protein kinase C α, β_I and β_{II} from cytosolic to membrane fractions in human neutrophils. However, celecoxib did not induce protein kinase C γ translocation from the cytosol to the cell membrane. According to this result, protein kinase C α, β_I and β_{II} could be involved in the free radical generation caused by celecoxib. In addition, Go-6976 (α and β_I inhibitor) and Ro-31-8220 (β_{II} inhibitor) significantly inhibited the free radical generation induced by celecoxib, indicating that protein kinase C α, β_I and β_{II} are involved in this process. Rottlerin did not inhibit the free radical generation induced by celecoxib, indicating that protein kinase C δ could not be involved in or only had a minor effect on the free radical release process induced by celecoxib. Moreover, protein kinase C ζ also moved from the cytosol to the cell membrane in the presence of celecoxib (data not shown). However, Go-6976 and Ro-31-8220 inhibited the superoxide anion release induced by celecoxib, and thus we suggest that protein kinase C α, β_I and β_{II} rather than δ and ζ play a major role in the free radical generation caused by celecoxib.

Chemotactic peptides, like fMLP, elicit superoxide anion production in neutrophils through the activation of NADPH oxidase. The role of calcium as a primary or secondary messenger in the neutrophil activation induced by fMLP has been extensively studied. The absence of extracellular calcium or depletion of intracellular calcium stores decreases fMLP-induced neutrophil superoxide anion production (Honeycutt and Niedel, 1986; Korchak et al., 1988). However, the role of an increase in intracellular calcium in fMLP-induced superoxide production remains unknown. BAPTA/AM inhibited celecoxib-induced free radical generation, indicating that an increase in intracellular calcium by celecoxib is necessary for free radical generation. The increase in intracellular calcium also implied that conventional protein kinase C was involved in celecoxib-induced superoxide anion release. According to the results showing that celecoxib increased intracellular calcium and activated protein kinase C, celecoxib may stimulate the signal upstream of calcium and protein kinase C. To evaluate this, we used pertussis toxin.

Pertussis toxin blocks the GTPase activity of G-proteins coupled to fMLP receptors in neutrophils (McLeish et al., 1989). The pertussis toxin-sensitive α_i G-protein subunit is the subunit most closely linked to the activation of N-formyl peptide receptors (Bokoch and Gilman, 1984). Pertussis toxin could not completely decrease the fMLP-induced increase in intracellular calcium in our experiment. Ruotsalainen and Savolainen (1995) reported similar results. They showed that both pertussis toxin-sensitive and cholera toxin-sensitive G-proteins were involved in the fMLP-mediated calcium increase and superoxide anion release. In our studies, pertussis toxin significantly inhibited superoxide anion production from celecoxib-stimulated neutrophils, suggesting that G-proteins are required in oxidative secretion pathways activated by celecoxib. Although pertussis toxin could not completely inhibit the superoxide anion release caused by celecoxib, this finding is consistent with information that pertussis toxin only partially blocks the intracellular calcium mobilization induced by celecoxib. The data suggest that celecoxib could induce intracellular calcium and superoxide anion release through pertussis toxin-sensitive G-

proteins and by other mechanisms. In our experiments, pertussis toxin decreased fMLP-induced membrane sequestration of protein kinase C (data not shown), indicating that the activation of protein kinase C is mediated by a G-protein-coupled receptor. Under the same conditions, pertussis toxin also partially decreased celecoxib-induced protein kinase C α, β_I and β_{II} translocation and intracellular calcium mobilization, indicating that activation of the second messengers by celecoxib required a pertussis toxin-sensitive G-protein-coupled receptor. Thus, celecoxib may activate a pertussis toxin-sensitive G-protein, resulting in an increase in intracellular calcium and protein kinase C activation. Intracellular calcium and protein kinase C synergistically release superoxide anion from human neutrophils. More experiments are needed to evaluate the direct or indirect effect of celecoxib on pertussis toxin-sensitive G-protein.

According to previous studies, tumor necrosis factor-α (TNF-α) up-regulates β_2-integrin in human neutrophils by increasing superoxide anion release and by activating tyrosine kinase and MAP kinase (Blouin et al., 1999; Tandon et al., 2000). In our study, the up-regulation of β_2-integrin expression by celecoxib was significantly inhibited by superoxide dismutase and genistein. Thus celecoxib-induced superoxide anion could modulate β_2-integrin expression through tyrosine kinase. BAPTA/AM decreased β_2-integrin expression caused by celecoxib in this study. A possible explanation is that BAPTA/AM decreased the superoxide anion release induced by celecoxib and then affected β_2-integrin expression indirectly. However, calcium is vital for β_2-integrin expression (Fekdhaus et al., 2002). Therefore, the decrease in intracellular calcium induced by BAPTA/AM could affect β_2-integrin expression directly. PD98059 inhibited the celecoxib-induced up-regulation of β_2-integrin expression, indicating that MAP kinase was activated and involved in the process of β_2-integrin expression caused by celecoxib in human neutrophils. In our study, superoxide dismutase and genistein inhibited celecoxib-induced MAP kinase activity, indicating that superoxide anion and tyrosine kinase affected MAP kinase activity. According to these data, celecoxib-induced superoxide anion could regulate β_2-integrin expression through activation of tyrosine kinase and MAP kinase.

In this study, we proved that celecoxib stimulates the production of superoxide anion in neutrophils by activating pertussis toxin sensitive G-protein, followed by an increase in intracellular calcium and protein kinase C activation. Furthermore, superoxide anion released from neutrophils may up-regulate β_2-integrin expression. Whether celecoxib increases neutrophil adhesion is under evaluation.

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