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Inhibition of superoxide anion and elastase release in human neutrophils by 3′-isopropoxychalcone *via* a cAMP-dependent pathway

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1 Chalcone is abundantly present in the plant kingdom and has various biological activities such as anti-inflammatory and antioxidant. In this study, the semisynthetic chalcone derivative, 3′-isopropoxychalcone (H2O7D), was demonstrated to inhibit the generation of superoxide and the release of elastase, as well as to accelerate resequestration of cytosolic calcium in formyl-L-methionyl-L-leucyl-L-phenylalanine-activated human neutrophils.

2 H2O7D displayed no antioxidant or superoxide-scavenging ability, and it failed to alter the subcellular NADPH oxidase activity.

3 H2O7D induced a substantial increase in cAMP but not cGMP levels. The elevation of cAMP formation by H2O7D was inhibited by adenosine deaminase (ADA). Furthermore, The inhibitory effects of H2O7D were reversed by protein kinase (PK)A inhibitors, as well as ADA and a selective A2a-receptor antagonist.

4 H2O7D inhibited phosphodiesterase (PDE) activities, but it did not alter adenylyl cyclase and soluble guanylyl cyclase activities. These results show that the cAMP-elevating effect of H2O7D results from the inhibition of PDE activity and not from the stimulation of cyclase function. Consistent with this, H2O7D potentiated the PGE_1-caused inhibitory effects and cAMP formation.

5 In summary, these results indicate that the inhibitory effect of H2O7D is cAMP/PKA dependent, and that it occurs through inhibition of cAMP PDE, which potentiates the autocrine functions of endogenous adenosine. Inhibition of respiratory burst and degranulation in human neutrophils may give this drug the potential to protect against the progression of inflammation.
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Abbreviations: AC, adenylyl cyclase; cAMP, cyclic adenosine 3′,5′-monophosphate; cGMP, cyclic guanosine 3′,5′-monophosphate; FMLP, formyl-L-methionyl-L-leucyl-L-phenylalanine; H89, *N*-(2-((*p*-bromocinnamyl)amino)ethyl)-5-isoquinolinesulfonamide; $O_2^{\bullet-}$, superoxide anion; PDE, phosphodiesterase; PKA, protein kinase A; LDH, lactate dehydrogenase; PMA, phorbol myristate acetate; sGC, soluble guanylyl cyclase; SOD, superoxide dismutase; Ro318220, 3-(1-(3-(amidinothio)propyl-1*H*-indol-3-yl))-3-(1-methyl-1*H*-indol-3-yl)maleimide; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt

Introduction

Chalcone, a flavonoid, is abundantly present in the plant kingdom and has various biological activities such as anti-inflammatory, antiallergic, antioxidant, antibacterial effects, and anticancer (Busse *et al*., 1984; Middleton & Drzewiecki, 1984; Lopez *et al*., 2001; Dominguez *et al*., 2005; Zi *et al*., 2005). In particular, a series of chalcone derivatives has been reported to have potent anti-inflammatory activities (Batt *et al*., 1993; Madan *et al*., 2000; Won *et al*., 2005). For example, chalcone derivatives exhibit potent inhibitory effects on the release of β-glucuronidase and lysozyme from rat neutrophils stimulated with formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)/cytochalasin B (CB) and on the production of nitric oxide and tumor necrosis factor-α from lipopolysaccharide-stimulated macrophages (Ko *et al*., 2003; Ban *et al*., 2004). In addition, chalcone derivatives show remarkable inhibitory effects on hind-paw edema induced by polymyxin B in normal as well as in adrenalectomized mice (Hsieh *et al*., 1998). In a search for new anti-inflammatory agents, the effect of semisynthetic chalcone derivatives on superoxide anion ($O_2^{\bullet-}$) release by human neutrophils was tested. Among them, 3′-isopropoxychalcone (H2O7D) showed the most-potent inhibitory effect on $O_2^{\bullet-}$ production in FMLP/CB-activated human neutrophils.

Neutrophils are known to play important roles in a host's defenses against invasion by microorganisms and in the pathogenesis of various diseases such as rheumatoid arthritis, ischemia–reperfusion injury, chronic obstructive pulmonary disease, and asthma (Malech & Gallin, 1987; Witko-Sarsat *et al*., 2000; Okajima *et al*., 2002; Ennis, 2003; Vinten-Johansen, 2004). In response to diverse stimuli, activated neutrophils secrete a series of cytotoxins, such as the $O_2^{\bullet-}$, a precursor of

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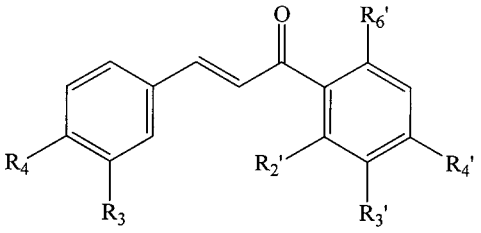
other reactive oxygen species (ROS), granule proteases, and bioactive lipids (Borregaard, 1998; Witko-Sarsat *et al*., 2000; Roos *et al*., 2003). Suppression of the extensive or inappropriate activation of neutrophils using drugs has been proposed as a way to ameliorate these inflammatory diseases. However, the pharmacological activities and target mechanisms of chalcone derivatives in human neutrophils are still unclear.

It is well established that cAMP is a key second messenger in regulating neutrophil functions (Coffey, 1992; Flamand *et al*., 2002; O'Dowd *et al*., 2004). cAMP is formed from ATP by the action of the enzyme, adenylyl cyclase (AC), and is degraded by a family of cAMP-specific phosphodiesterase (PDE) enzymes, which catalyzes the hydrolysis of cAMP to inactive 5′-AMP. Elevation of intracellular cAMP levels is believed to suppress the activation of neutrophils. For example, adenosine, which activates the Gαs protein to stimulate AC *via* occupancy of A2a receptors on neutrophils, has been widely recognized to diminish the inflammatory response (Flamand *et al*., 2000; Harada *et al*., 2000). Since the predominant PDE in most inflammatory cells belongs to the PDE4 family, the inhibitors of PDE4 are being developed clinically as potential anti-inflammatory agents. The clinical potential of cAMP-elevating agents as inhibitors of neutrophil activities is supported by the suppression of endotoxin-induced acute lung injury in mice by the PDE inhibitor, rolipram (Miotla *et al*., 1998), and the anti-inflammatory activity of the new-generation PDE inhibitors, SB 207499 and AWD 12-281, in experimental asthma and chronic obstructive pulmonary disease (Underwood *et al*., 1998; Kuss *et al*., 2003). In the present study, the mechanisms of action of 3′-isopropoxychalcone were further investigated. Our data suggest that inhibition of human neutrophil functions by H2O7D is cAMP dependent.



Compounds	R_2'	R_3'	R_4'	R_6'	R_3	R_4
H2O7A	H	H	OH	H	H	H
H2O7B	H	H	O*i*Pr	H	H	H
H2O7C	H	OH	H	H	H	H
H2O7D	H	O*i*Pr	H	H	H	H
H2O7E	OH	H	H	H	H	H
H2O7F	O*i*Pr	H	H	H	H	H
H2O8A	H	H	OH	H	OH	H
H2O8B	H	OH	H	H	H	OH
H2O8C	H	OH	H	H	OH	H
H2O8D	OH	H	H	H	OH	H
H2O9A	O*i*Pr	H	OCH_3	OH	H	H
H2O9B	OCH_3	H	OCH_3	OH	H	H
H2O9C	OH	H	OCH_3	OH	H	H

Figure 1 Chemical structures of the chalcone derivatives.

Methods

Materials

Chalcone derivatives (Figure 1) were synthesized (unpublished results) and were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions. Aprotinin, *N*-(2-((*p*-bromocinnamyl)-amino)ethyl)-5-isoquinolinesulfonamide (H89), KT5720 (9*S*,10*S*,12*R*-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo(1,2,3-fg:3′,2′,1′-kl)pyrrolo(3,4-i)(1,6)benzodiazocine-10-carboxylic acid hexyl ester), leupeptin, phenylmethylsulfonyl fluoride (PMSF), 3-(1-(3-(amidinothio)-propyl-1*H*-indol-3-yl))-3-(1-methyl-1*H*-indol-3-yl)maleimide (Ro318220), rolipram, and zaprinast were obtained from Calbiochem (La Jolla, CA, U.S.A.). Fluo-3 AM was purchased from Molecular Probes (Eugene, OR, U.S.A.). 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were obtained from Sigma (St Louis, MO, U.S.A.). When drugs were dissolved in DMSO, the final concentration of DMSO in cell experiments did not exceed 0.5% and did not affect the parameters measured.

Preparation of human neutrophils

Human neutrophils from venous blood of healthy, adult volunteers (male and female, 20–28 years old) were isolated with a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes (Boyum *et al*., 1991). Purified neutrophils that contained >98% viable cells, as determined by the trypan blue exclusion method, were resuspended in a calcium (Ca^{2+})-free HBSS buffer at pH 7.4, and were maintained at 4°C before use.

Neutrophil fractionation

Neutrophils were pretreated with 1 mM PMSF for 30 min at 4°C, disrupted in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, and 10 mM PIPES; pH 7.3) by sonication, and centrifuged at $100{,}000 \times g$ for 20 min at 4°C to produce cytosolic and plasma membrane fractions.

Measurement of $O_2^{\bullet-}$ generation

The assay of $O_2^{\bullet-}$ generation was based on the SOD-inhibitable reduction of ferricytochrome *c* (Babior *et al*., 1973). In brief, after supplementation with 0.5 mg ml^{-1} ferricytochrome *c* and 1 mM Ca^{2+}, neutrophils ((4 or 10) $\times 10^5$ ml^{-1}) were equilibrated at 37°C for 2 min and incubated with drugs for 5 min. Cells were activated with FMLP (100 nM) for 10 or PMA (2 nM) for 5 min. When FMLP was used as a stimulant, CB (1 μg ml^{-1}) was incubated for 3 min before activation by the peptide (FMLP/CB). $O_2^{\bullet-}$ generation by isolated neutrophil fractionation was measured after the addition of 160 μM NADPH to 800 μl of relaxation buffer containing 4×10^6 cell equivalents of membrane extract, 1.2×10^7 cell equivalents of cytosol, 2 μM GTP-γ-S, 0.5 mg ml^{-1} ferricytochrome *c*, and 100 μM sodium dodecyl sulfate. To

facilitate the assembly of NADPH oxidase components, all constituents (excluding NADPH) were incubated at 37°C for 3 min before the addition of NADPH. Drugs were incubated for 2 min before NADPH oxidase assembly. Changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010, Tokyo, Japan). Calculations were based on differences in the reactions with and without SOD (100 U ml^{-1}) divided by the extinction coefficient for the reduction of ferricytochrome *c* ($\varepsilon = 2.11\,\text{mM}^{-1}\,\text{mm}^{-1}$).

Measurement of ROS release

ROS were measured using a lucigenin-enhanced chemiluminescence (LECL) method. Neutrophils (3×10^5) were preincubated at 37°C for 5 min in 250 μl HBSS containing 30 μM lucigenin and incubated with drugs for 5 min. Cells were activated by FMLP (100 nM)/CB (1 μg ml^{-1}) in a preincubation with drugs for 5 min, and LECL responses were detected using a 96-well chemiluminometer (FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany).

LDH release

Cytotoxicity was expressed as the percent LDH activity obtained in cell-free medium compared to the total LDH activity. Total LDH activity was determined by lysing cells with 0.1% Triton X-100 for 30 min at 37°C.

$O_2^{\bullet-}$-scavenging activity

The $O_2^{\bullet-}$-scavenging ability of H2O7D was determined using xanthine/xanthine oxidase in a cell-free system, based on a previously described method (Tan & Berridge, 2000). After 0.1 mM xanthine was added to the assay buffer (50 mM Tris (pH 7.4), 0.3 mM WST-1, and 0.02 U ml^{-1} xanthine oxidase) for 15 min at 30°C, the absorbance associated with the $O_2^{\bullet-}$-induced WST-1 reduction was measured at 450 nm.

1,1-Diphenyl-2-picrylhydrazyl (DPPH)-scavenging activity

An ethanol solution of the stable nitrogen-centered free radical, DPPH (100 μM), was incubated with H2O7D or α-tocopherol for 16 min at 25°C, and the absorbance was measured at 517 nM.

Measurement of elastase release

Degranulation of azurophilic granules was determined by elastase release as described previously (Sklar *et al*., 1982; Coles *et al*., 2002) with some modifications. Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide as the elastase substrate. Briefly, after supplementation with MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (100 μM), neutrophils (5×10^5 ml^{-1}) were equilibrated at 37°C for 2 min and incubated with drugs for 5 min. Cells were activated by FMLP (100 nM)/CB (0.5 μg ml^{-1}), and changes in absorbance at 405 nm were continuously monitored to assay elastase release. The results were expressed as the percent of the initial rate of elastase release in the FMLP/CB-activated, drug-free control system.

Determination of cAMP and cGMP concentrations

cAMP and cGMP levels were assayed using enzyme immunoassay kits (Amersham Pharmacia Biotech, GE Healthcare Bio-Sciences, Little Chalfont, U.K.). The reaction of neutrophils was terminated by adding 0.5% dodecytrimethylammonium bromide. Samples were then centrifuged at $3000 \times g$ for 5 min at 4°C. The supernatants were used as a source for the cAMP and cGMP samples. The assay was performed according to the manufacturer's instructions.

Assay of AC, soluble guanylyl cyclase (sGC), and PDE activities

Neutrophils (5×10^7 cells ml^{-1}) were sonicated in ice-cold buffer, containing 25 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 2 mM EDTA, 5 mM $MgCl_2$, 10 μM leupeptin, 100 μM PMSF, and 10 μM pepstatin, and then cells were centrifuged at $100{,}000 \times g$ for 40 min at 4°C. The pellet and supernatant fraction were, respectively, used as sources for the AC and sGC or PDE enzymes. The reaction mixture (25 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine (IBMX), 7.5 mM creatine phosphate, and 3 U creatine phosphokinase) contained 0.5 mM dithiothreitol, 1 mM ATP, and the pellet fraction for assessing AC activity, or contained 1 mM GTP and the supernatant fraction for assessing sGC activity. The reaction was carried out for 20 min at 30°C and was terminated by boiling for 3 min. cAMP or cGMP contents were assayed using enzyme immunoassay kits.

PDE activity was analyzed using a tritium scintillation proximity assay (SPA) system, and the assay was performed according to the manufacturer's instructions (Amersham Pharmacia Biotech). Briefly, assays were performed at 30°C for 10 min in the presence of 50 mM Tris-HCl (pH 7.5) containing 8.3 mM $MgCl_2$, 1.7 mM EGTA, and 0.3 mg ml^{-1} bovine serum albumin. Each assay was performed in a 100-μl reaction volume containing the above buffer, the neutrophil supernatant fraction, and around 0.05 μCi [^{3}H]cAMP or [^{3}H]cyclic GMP (for cAMP- or cGMP-specific PDE activity, respectively). The reaction was terminated by the addition of 50 μl PDE SPA beads (1 mg) suspended in 18 mM zinc sulfate. Assays were performed in 96-well microtiter plates. The reaction mix was allowed to settle for 1 h before counting in a microtiter plate counter.

Measurement of $[Ca^{2+}]_i$

Neutrophils were loaded with 2 μM fluo-3 AM at 37°C for 45 min. After being washed, cells were resuspended in calcium-free HBSS to 3×10^6 cells ml^{-1}. The change in fluorescence was monitored using a Hitachi F-4500 spectrofluorometer (Tokyo, Japan) in a quartz cuvette with a thermostat (37°C), while being continuously stirred. The excitation wavelength was 488 nm, and the emission wavelength was 520 nm. FMLP was used to increase $[Ca^{2+}]_i$ in the presence of 1 mM Ca^{2+}. $[Ca^{2+}]_i$ was calibrated by the fluorescence intensity, as follows: $[Ca^{2+}]_i = K_d \times [(F - F_{min})\,(F_{max} - F)^{-1}]$; where F is the observed fluorescence intensity, F_{max} and F_{min} were, respectively, obtained by the addition of 0.05% Triton X-100 and 10 mM EGTA, and K_d was taken to be 400 nM.

Statistical analysis

Results are expressed as the mean ± s.e.m. Data were analyzed using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, U.S.A.). Statistical analysis was performed using Student's *t*-test or a repeated-measures one-way analysis of variance followed by Bonferroni's multiple comparison test when appropriate. A two-way analysis of variance followed by Bonferroni's multiple comparison test was used as required (for Figures 4, 5a, and 6). A value of $P<0.05$ was considered statistically significant.

Results

H2O7D inhibits FMLP/CB-induced $O_2^{\bullet-}$ generation by intact neutrophils but not by reconstituted NADPH oxidase

To investigate whether chalcone derivatives reduced respiratory burst by human neutrophils in response to FMLP/CB, the amount of $O_2^{\bullet-}$ was determined. Among them, H2O7D showed the most-potent inhibitory effect on $O_2^{\bullet-}$ production with an IC_{50} value of 0.24 ± 0.08 μM (Figure 2a and Table 1). H2O7D failed to alter the basal $O_2^{\bullet-}$ generation under resting conditions, whereas it inhibited $O_2^{\bullet-}$ release in FMLP/CB-treated human neutrophils in a concentration-dependent manner (Figure 2a). Culturing with H2O7D (up to 30 μM) did not affect cell viability, as assayed by LDH release. Rolipram (1 μM), a well-documented inhibitor of PDE4, inhibited FMLP/CB-induced $O_2^{\bullet-}$ release in human neutrophils. Additionally, H2O7D inhibited PMA-stimulated $O_2^{\bullet-}$ release at 3 μM. Ro318220 (1 μM), a well-documented inhibitor of protein kinase (PK)C, was used as a positive control on PMA-caused $O_2^{\bullet-}$ generation (Figure 2b). To examine whether NADPH oxidase was involved in the inhibition of H2O7D, neutrophil membranes were isolated to assay $O_2^{\bullet-}$ production in a reconstituted system after the addition of NADPH. As shown in Figure 2c, diphenyleneiodonium, but not H2O7D, suppressed $O_2^{\bullet-}$ generation. These data indicate that H2O7D does not inhibit $O_2^{\bullet-}$ release through direct inhibition of NADPH oxidase activity.

$O_2^{\bullet-}$- and free radical-scavenging activity of H2O7D

To investigate the ability of H2O7D to scavenge $O_2^{\bullet-}$ and free radicals, the effects of H2O7D in the cell-free xanthine/xanthine oxidase system and DPPH test were assayed. H2O7D, at concentrations of up to 30 μM, failed to alter the xanthine/xanthine oxidase-induced WST-1 reduction and the stability of DPPH radicals (Figure 3). SOD and α-tocopherol were used as the positive controls in the xanthine/xanthine oxidase system and DPPH assay, respectively. Additionally, H2O7D did not affect the removal of $O_2^{\bullet-}$ by SOD (0.5 U ml^{-1}) in the xanthine/xanthine oxidase system (Figure 3a). These data rule out the possibility that the inhibitory effect of H2O7D on $O_2^{\bullet-}$ release occurs through scavenging of $O_2^{\bullet-}$and free radicals.

PKA mediates the inhibition of FMLP/CB-stimulated $O_2^{\bullet-}$ release by H2O7D

To examine whether cAMP is involved in the inhibitory effect of H2O7D, pharmacological agents were used to elucidate the

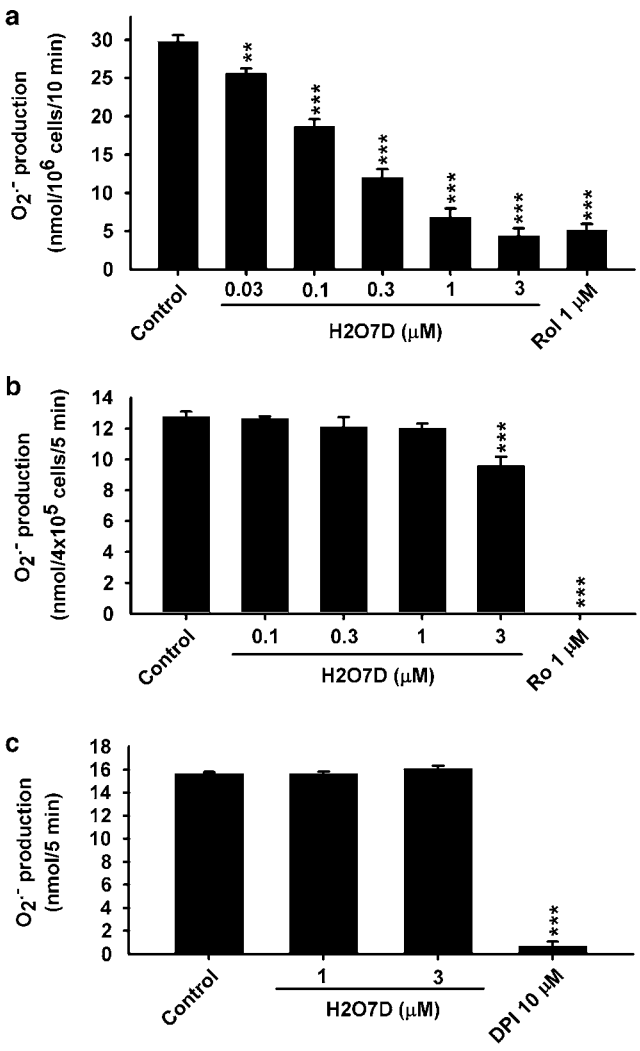


Figure 2 Effects of H2O7D on $O_2^{\bullet-}$ generation in FMLP/CB- or PMA-activated human neutrophils, and in isolated neutrophil membranes. $O_2^{\bullet-}$ generation was measured using SOD-inhibitable cytochrome *c* reduction, as described under Methods. Human neutrophils were incubated with DMSO (control), H207D (0.03–3 μM), rolipram (Rol, 1 μM), or Ro318220 (Ro, 1 μM) for 5 min and then activated by FMLP/CB ($n=6$) (a) or PMA ($n=3$) (b). (c) A reactive mixture of the neutrophil cytosolic fraction and membrane fraction was preincubated with DMSO, H2O7D (1 and 3 μM), or diphenyleneiodonium (DPI, 10 μM) at 37°C for 2 min before the addition of sodium dodecyl sulfate (100 μM). The reaction was initiated by adding 160 μM NADPH ($n=3$). All data are expressed as the mean ± s.e.m. $^{**}P<0.01$; $^{***}P<0.001$ compared with the control.

mechanisms. The PKA inhibitors, H89 (3 μM) and KT5720 (0.3 μM), reduced the inhibition of FMLP/CB-stimulated $O_2^{\bullet-}$ formation by H2O7D and rolipram (Figure 4). These results suggest that PKA mediates the inhibition of FMLP/CB-stimulated $O_2^{\bullet-}$ generation caused by H2O7D in human neutrophils. Furthermore, the adenosine deaminase (ADA, 2 U ml^{-1}) and selective A2a-receptor antagonist, 8-(*p*-sulfophenyl)theophylline (30 μM), also reduced the inhibition of FMLP/CB-stimulated $O_2^{\bullet-}$ formation by H2O7D, rolipram, and adenosine. In contrast, ADA and 8-(*p*-sulfophenyl)theophylline failed to alter the PGE_1-caused inhibition (Figure 5a). Furthermore, H2O7D markedly potentiated PGE_1-induced inhibition in the presence of ADA (Figure 5b).

Table 1 Effects of chalcone derivatives on $O_2^{\bullet-}$ generation by human neutrophils in response to FMLP/CB

Compounds	*IC_{50}* (μM)[a]
H2O7A	6.04±1.86
H2O7B	(84.81±4.52)[b]
H2O7C	3.63±0.58
H2O7D	0.24±0.08
H2O7E	0.92±0.40
H2O7F	3.58±0.92
H2O8A	9.59±0.81
H2O8B	6.04±0.55
H2O8C	4.52±0.47
H2O8D	3.65±0.66
H2O9A	>30
H2O9B	(73.54±1.93)[b]
H2O9C	0.63±0.26

All data are presented as the mean±s.e.m. ($n=3$ or 4).
[a]Concentration necessary for 50% inhibition (IC_{50}).
[b]H2O7B- and H2O9B-elicited human neurophil $O_2^{\bullet-}$ generation in the absence of FMLP/CB.
Results are shown as the percent of FMLP/CB without compounds.

H2O7D inhibits FMLP/CB-induced ROS release

The $O_2^{\bullet-}$ formed in neutrophils can be converted to various species of oxygen radicals that are strongly antimicrobial but which also directly or indirectly cause damage by destroying the surrounding tissue. As shown in Figure 6a, H2O7D, rolipram, and adenosine inhibited ROS release by human neutrophils in response to FMLP/CB. These inhibitory effects were abolished by the PKA inhibitor H89 (Figure 6a). SOD (100 U ml^{-1}) was used as a positive control and completely repressed the ELCL responses.

H2O7D inhibits FMLP/CB-induced elastase release

Neutrophil degranulation was measured according to the extent of release of the primary granule-derived protease, elastase. H2O7D and rolipram inhibited elastase release by human neutrophils in response to FMLP/CB (Figure 6b). On the other hand, H2O7D did not alter the basal level of elastase release under resting conditions (data not shown). The PKA inhibitor H89 restored the inhibition of H2O7D and rolipram (Figure 6b). These results suggest that cAMP/PKA also mediates the H2O7D-caused inhibition of degranulation in human neutrophils.

Effect of H2O7D on cAMP formation and AC activity

cAMP concentrations were measured to determine whether the inhibitory effects of H2O7D are likely to be associated with cAMP. H2O7D (3 and 10 μM) and PGE_1 (1 and 3 μM) notably increased cAMP levels in FMLP-stimulated human neutrophils (Figure 7a). Elevations of cAMP levels by H2O7D, but not by PGE_1, were abolished by ADA. Furthermore, in the presence of ADA, H2O7D significantly potentiated PGE_1-induced cAMP formation (Figure 7a). Cellular cAMP concentrations are modulated either by synthesis *via* AC or by degradation *via* PDEs. Our data showed that forskolin (30 μM), but not H2O7D (1, 3, and 10 μM), increased the activity of AC (Figure 7b). On the other hand, neither sGC activity nor cGMP concentration was changed by H2O7D (data not shown).

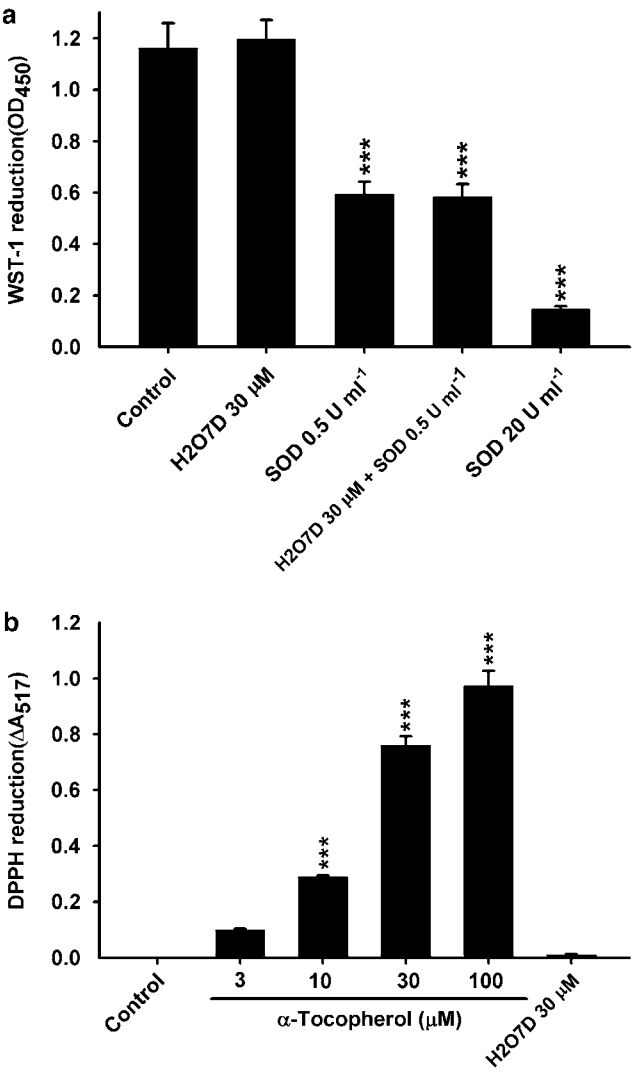


Figure 3 Antioxidant effects of H2O7D in a cell-free xanthine/xanthine oxidase system and DPPH assay. Reduction of WST-1 ($n=3$) (a) and DPPH ($n=4$) (b) was, respectively, measured spectrophotometrically at 450 and 517 nm, as described under Methods. All data are expressed as the mean±s.e.m. ***$P<0.001$ compared with the control.

Effect of H2O7D on PDE activity

As shown in Figure 8, H2O7D inhibited cAMP-specific PDE and cGMP-specific PDE in a concentration-dependent manner. H2O7D was more effective at inhibiting cAMP-specific PDE than cGMP-specific PDE. Rolipram (1–30 μM), a PDE4 inhibitor, and zaprinast (0.1, 1, and 10 μM), a PDE5 inhibitor, were used as positive controls for inhibiting cAMP-specific PDE and cGMP-specific PDE, respectively. IBMX, a nonselective PDE inhibitor, inhibited cAMP and cGMP PDEs. Moreover, the combination of H2O7D and rolipram did not further inhibit cAMP-specific PDE (Figure 8a).

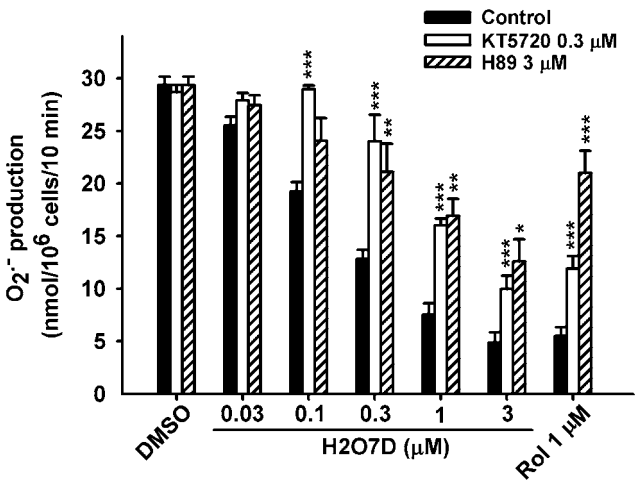


Figure 4 Effects of PKA inhibitors on H2O7D-caused inhibition of $O_2^{\bullet-}$ generation in human neutrophils. H89 (3 μM) and KT5720 (0.3 μM) were preincubated for 5 min before the addition of H2O7D (0.03–3 μM) or rolipram (Rol, 1 μM). $O_2^{\bullet-}$ generation was induced by FMLP/CB and measured using SOD-inhibitable cytochrome *c* reduction, as described under Methods. All data are expressed as the mean$\pm$s.e.m. ($n=5$). $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ compared with the corresponding control.

Effect of H2O7D on $[Ca^{2+}]_i$

Peak $[Ca^{2+}]_i$ values were unaltered by H2O7D and rolipram in FMLP-induced human neutrophils, but the time it took for $[Ca^{2+}]_i$ to return to half of the peak values ($t_{1/2}$) was significantly shortened (Table 2). H89 restored the inhibition of $t_{1/2}$ by H2O7D and rolipram. Additionally, the combination of PGE_1 (0.01 μM) with either H2O7D (1 μM) or rolipram (0.03 μM) further reduced $t_{1/2}$ values. Furthermore, H89 alone significantly increased FMLP-induced $t_{1/2}$ values (from 26.37 ± 1.31 to 36.47 ± 4.61 s; $P<0.01$) (Table 2), implying that cAMP feeds back to inhibit resequestration of $[Ca^{2+}]_i$ in FMLP-stimulated human neutrophils.

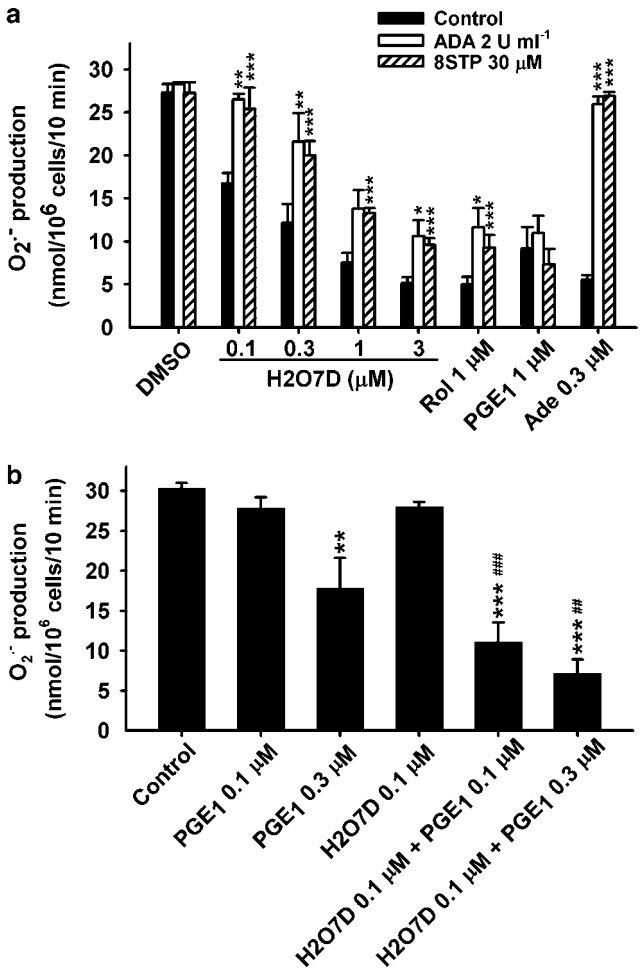


Figure 5 Effects of ADA and an A2a-receptor antagonist on the inhibition of neutrophil $O_2^{\bullet-}$ generation by H2O7D with or without PGE_1. (a) ADA (2 U ml^{-1}) and 8-(*p*-sulfophenyl)theophylline (8STP, 30 μM) were preincubated for 5 min before the addition of H2O7D (0.1–3 μM), rolipram (Rol, 1 μM), PGE_1 (1 μM), or adenosine (Ade, 0.3 μM). (b) Inhibition of $O_2^{\bullet-}$ generation by H2O7D with PGE_1 in the presence of ADA. H2O7D (0.1 μM) was tested with or without PGE_1 (0.1 and 0.3 μM) in the presence of ADA (2 U ml^{-1}). $O_2^{\bullet-}$ generation was induced by FMLP/CB and measured using SOD-inhibitable cytochrome *c* reduction, as described under Methods. All data are expressed as the mean$\pm$s.e.m. ($n=3$–5). $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ compared with the corresponding control. $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ compared with the corresponding PGE_1.

Discussion

In the present study, we investigated the effects of chalcone derivatives on respiratory burst and degranulation in human neutrophils, which play an important role in the pathogenesis of rheumatoid arthritis, ischemia–reperfusion injury, chronic obstructive pulmonary disease, asthma, and other inflammatory processes (Malech & Gallin, 1987; Witko-Sarsat *et al*., 2000; Okajima *et al*., 2002; Ennis, 2003; Vinten-Johansen, 2004). Among these compounds, H2O7D is the most-powerful inhibitory agent. H2O7D inhibited the generation of $O_2^{\bullet-}$ and the release of elastase, as well as accelerated resequestration of cytosolic calcium in FMLP-activated human neutrophils in a concentration-dependent fashion. Investigation of the signal transduction pathways indicates that the inhibitory effects of H2O7D are closely associated with elevation of cAMP concentrations.

Stimulation of neutrophils leads to increases in their oxygen consumption through the activity of NADPH oxidase which generates $O_2^{\bullet-}$. This phenomenon is the so-called respiratory burst (Dahlgren & Karlsson, 1999). The activated NADPH oxidase is a membrane-bound enzyme complex. Upon stimulation, the cytosolic components, including $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small GTPase Rac2, translocate to the membrane, where they associate with flavocytochrome b_{558}, which consists of $gp91^{phox}$ and $p22^{phox}$, to form the catalytically active oxidase (Jones *et al*., 2000; Roos *et al*., 2003). $O_2^{\bullet-}$ production is linked to the killing of invading microorganisms, but it can also directly or indirectly cause damage by destroying surrounding tissue. The formation of $O_2^{\bullet-}$ in neutrophils can be inhibited by modulating the cellular signaling pathways, but also by directly scavenging $O_2^{\bullet-}$. H2O7D at concentrations of up to 30 μM did not scavenge $O_2^{\bullet-}$ or DPPH radicals in cell-free systems, indicating that the inhibitory effect of H2O7D on $O_2^{\bullet-}$ release does not occur through the scavenging of $O_2^{\bullet-}$ and free radicals.

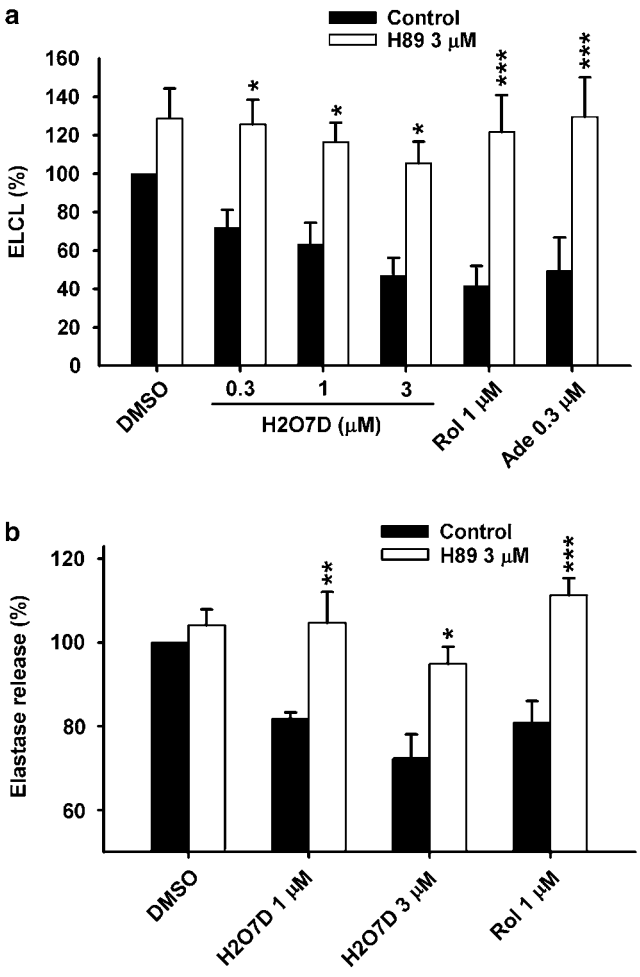


Figure 6 Effects of a PKA inhibitor on the inhibition of ROS generation and elastase release by H2O7D in human neutrophils. H89 (3 μM) was preincubated for 5 min before the addition of H2O7D (0.3, 1, and 3 μM), rolipram (Rol, 1 μM), or adenosine (Ade, 0.3 μM). ROS generation ($n=4$) (a) and elastase release ($n=3$) (b) were induced by FMLP/CB and, respectively, measured using the ELCL method and spectrophotometrically at 405 nm, as described under Methods. All data are expressed as the mean$\pm$s.e.m. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ compared with the corresponding control.

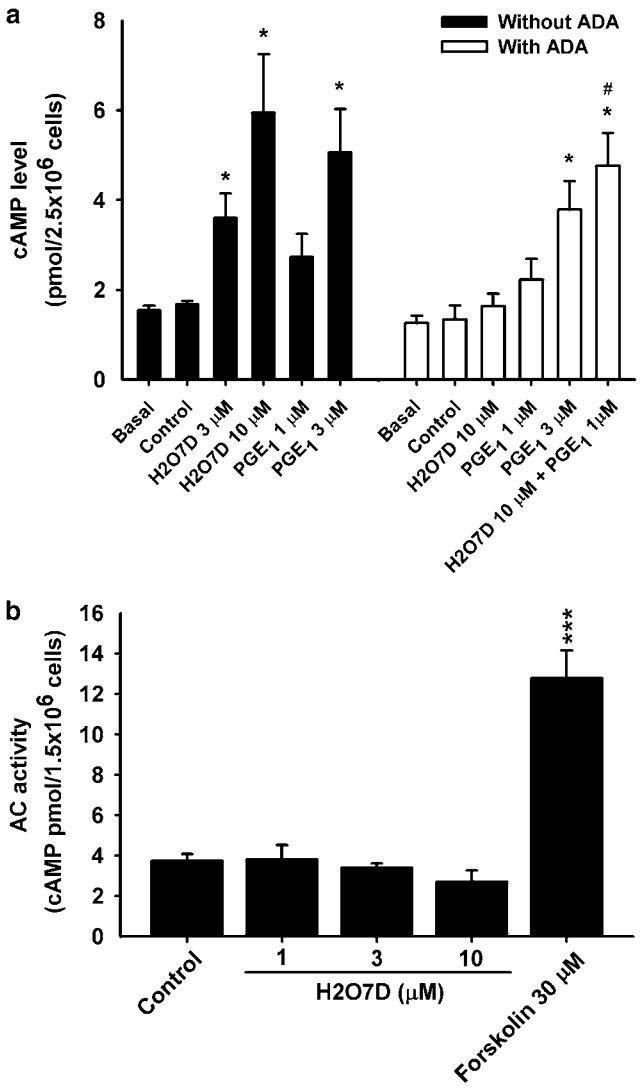


Figure 7 Effects of H2O7D on cAMP level and AC activity. (a) Human neutrophils were incubated with H2O7D (3 and 10 μM), PGE_1 (1 and 3 μM), and H2O7D with PGE_1 for 5 min before stimulation with FMLP for another 5 min in the presence or absence of ADA (2 U ml^{-1}). (b) Neutrophil membrane fractions were incubated with H2O7D (1, 3, and 10 μM) and forskolin (30 μM) at 30°C for 20 min in the presence of 1 mM ATP. cAMP was assayed using enzyme immunoassay kits. All data are expressed as the mean$\pm$s.e.m. ($n=3$). $^{*}P<0.05$; $^{***}P<0.001$ compared with the corresponding control. $^{\#}P<0.05$ compared with the corresponding H2O7D.

The lack of inhibition of NADPH oxidase by H2O7D shows that H2O7D exerts its inhibitory influence upstream of NADPH oxidase.

Increases in intracellular cAMP concentrations in neutrophils are associated with a decrease in several neutrophil functions, including respiratory burst, degranulation, and release of bioactive lipids (Anderson *et al*., 1998; Flamand *et al*., 2002; Hwang *et al*., 2003). Our results are in line with previous findings that various cAMP-elevating agents can suppress $O_2^{\bullet-}$ generation and elastase release stimulated by FMLP. H2O7D significantly elevated cAMP concentrations in human neutrophils. Two structurally different PKA inhibitors, H89 and KT5720, reduced the H2O7D-induced inhibition. These results indicate that PKA mediates the inhibition of respiratory burst and degranulation by H2O7D. Adenosine, generated by dephosphorylation of adenylates, is well accepted as an important physiological modulator of the proinflammatory activities of human neutrophils (Cronstein, 1994). The anti-inflammatory properties of adenosine appear to be mediated through interactions with Gαs protein/AC-coupled adenosine receptors of the A2a receptor subtype (Thibault *et al*., 2002). Based on the observations that ADA and the selective A2a receptor antagonist, 8-(*p*-sulfophenyl)theophylline, reduced H2O7D- but not PGE_1-caused inhibition of $O_2^{\bullet-}$ generation and elevation of cAMP formation, we propose that the autocrine inhibitory actions of endogenous adenosine are enhanced by H2O7D. Consistent with this hypothesis that rolipram-mediated effect was also inhibited by ADA and 8-(*p*-sulfophenyl)theophylline. Alternatively, H2O7D failed to alter sGC activity and the cGMP concentration. These data rule out a role for cGMP in H2O7D-caused inhibition.

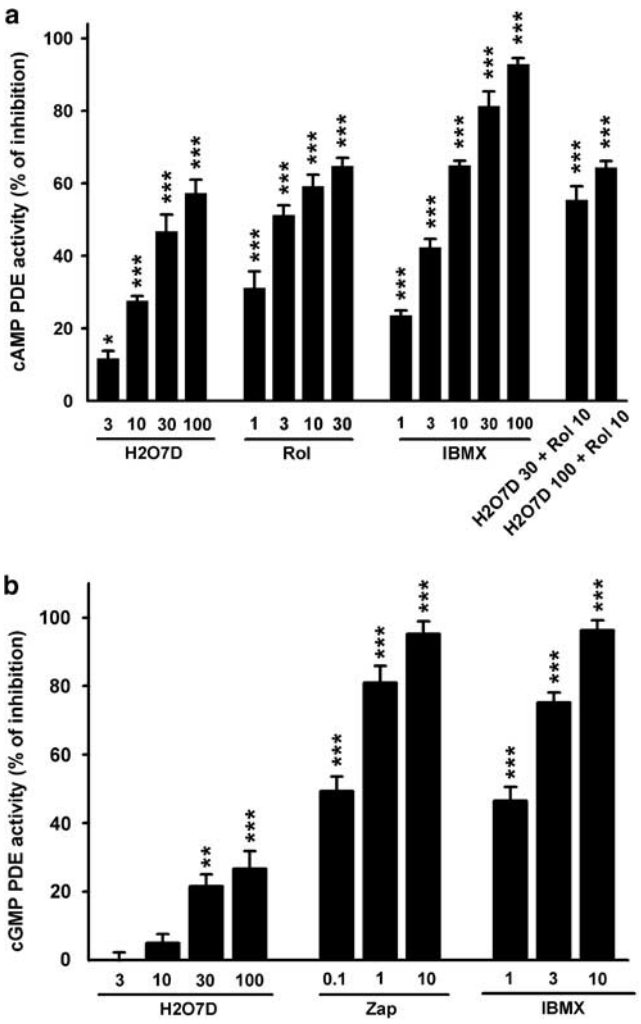


Figure 8 Concentration-dependent effects of H2O7D on the inhibition of cAMP and cGMP PDE activities. Human neutrophil homogenates were incubated with H2O7D (3–100 μM), rolipram (Rol, 1–30 μM), zaprinst (Zap, 0.1, 1, and 10 μM), IBMX (1–100 μM), or H2O7D (30 and 100 μM) with Rol (10 μM), and then 0.05 μCi [^{3}H]cAMP (a) or [^{3}H]cGMP (b) was added to the reaction mixture at 30°C for 10 min. PDE activity was measured as described under Methods. All data are expressed as the mean ± s.e.m. ($n = 3$–5). *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with the control.

Table 2 Effects of H2O7D and rolipram, with or without H89 or PGE_1, on the peak $[Ca^{2+}]_i$ and the time taken for this concentration to decline to half of its peak value ($t_{1/2}$) in FMLP-activated neutrophils

Drug	*Peak* $[Ca^{2+}]_i$ (nM)	$T_{1/2}$ (s)
Control	301.20 ± 8.57	26.37 ± 1.31
H2O7D, 1 μM	294.44 ± 9.33	20.61 ± 1.99**
H2O7D, 3 μM	285.14 ± 7.46	16.16 ± 0.87***
Rolipram, 0.03 μM	314.33 ± 14.50	23.83 ± 1.92
Rolipram, 3 μM	285.83 ± 10.99	12.10 ± 0.96***
H89, 5 μM	299.17 ± 6.63	36.47 ± 4.61**
H89, 5 μM + H2O7D, 3 μM	308.00 ± 11.02	35.72 ± 3.75**
H89, 5 μM + rolipram, 3 μM	319.50 ± 12.02	37.32 ± 5.30**
PGE_1, 0.01 μM	305.33 ± 20.22	27.77 ± 0.58
PGE_1, 0.01 μM + H2O7D, 1 μM	319.67 ± 16.05	12.40 ± 0.70***
PGE_1, 0.01 μM + rolipram, 0.03 μM	319.33 ± 19.01	14.30 ± 1.50***

*$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with the control.

It is well established that Ca^{2+} signaling is a key second messenger in regulating neutrophil functions (Harfi *et al*., 2005). Stimulation of GPCR induces the Ca^{2+} signal *via* activation of phospholipase C, which hydrolyses phosphatidylinositol 4,5-bisphosphate into IP_3 and diacylglycerol. IP_3 triggers rapid Ca^{2+} release from internal Ca^{2+} stores by activating IP_3 receptors and a consequent transient increase in $[Ca^{2+}]_i$ as the initial phase, which is followed by sustained $[Ca^{2+}]_i$ changes (Berridge, 1993). Neither H2O7D nor rolipram altered the FMLP-induced peak Ca^{2+}, but they did accelerate the resequestration of cytosolic Ca^{2+}, consistent with previous findings that cAMP increases the clearance of Ca^{2+} from the cytosol (Tintinger *et al*., 2001). Likewise, this phenomenon was inhibited by H89 and enhanced by PGE_1. cAMP inhibition of FMLP- but not PMA-induced $O_2^{\bullet-}$ generation by neutrophils has been reported (Sedgwick *et al*., 1985; Hwang *et al*., 2003). H2O7D at higher concentrations inhibited PMA-activated $O_2^{\bullet-}$ release by neutrophils. In addition, PKA inhibitors did not completely restore the H2O7D-induced inhibition. These data suggest that H2O7D at higher concentrations may exhibit an additional cAMP-independent mechanism of action. The various flavonids have been reported to inhibit other signaling pathways. For example, butein inhibited phosphorylation of p42/44 MAP kinase and degradation of IκB in lipopolysaccharide-stimulated macrophages (Lee *et al*., 2004), and quercetin was shown to be an inhibitor of phosphorylase kinase and also of protein tyrosine kinase (Srivastava, 1985). Therefore, the additional mechanism of action involving in H2O7D-mediated inhibition in human neutrophils remains to be established.

Cyclic nucleotide levels may be regulated by either production or degradation. Our data showed that H2O7D did not increase AC's functions, but attenuated PDE's activities. This result shows that the cAMP-elevating effect of H2O7D results from the inhibition of PDE activity and not from the stimulation of cyclase function. Consistent with this, H2O7D potentiated the PGE_1-caused inhibitory effects and cAMP formation. Interestingly, the combination of H2O7D and rolipram did not further inhibit cAMP-specific PDE, suggesting that H2O7D inhibits the breakdown of cAMP by rolipram-sensitive PDE. The maximum inhibition of rolipram on cAMP-specific PDE was only about 60%, meaning that neutrophils contain a variety of different cAMP-specific PDE isozymes. Neutrophils possess four PDE isozymes, subtypes PDE3, PDE4, PDE5, and PDE7 (VanUffelen *et al*., 1998; Schudt *et al*., 1991; Smith *et al*., 2003). Clearly, additional work is required to define the selectivity of H2O7D on PDE isozymes.

In summary, the present study shows that H2O7D inhibits human neutrophil proinflammatory responses, including respiratory burst, degranulation, and calcium mobilization. These effects are attributed to the elevation of cellular cAMP through the inhibition of cAMP-specific PDE.

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