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PROSTAGLANDIN E_1 AND PROSTAGLANDIN I_2 MODULATION OF SUPEROXIDE PRODUCTION BY HUMAN NEUTROPHILS

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SUMMARY: 15(S)-15-methyl-prostaglandin E_1 and prostaglandin I_2 rapidly and reversibly inhibit formyl-methionyl-leucyl-phenylalanine induced superoxide production by human neutrophils. In contrast, 15(S)-15-methyl-prostaglandin E_1 and prostaglandin I_2 did not alter the rate or the total amount of superoxide production by human neutrophils stimulated with either phorbol myristate acetate or arachidonic acid. These data suggest that the production of superoxide anion by human neutrophils may be mediated by at least two mechanisms, one regulated by prostaglandins and intracellular cyclic adenosine monophosphate levels and a second independent of prostaglandin modulation.

Polymorphonuclear leukocytes (PMNs) are a primary cellular component of acute inflammatory reactions and have been shown to cause cell and tissue injury (reviewed 1). One of the principal products of their activation is the production of superoxide anion (O_2^-) which is generated by a membrane-associated nicotinamide adenine dinucleotide oxidase of which the cofactor binding site has greater affinity for NADPH (2,3). This oxidase system frequently referred to as the NADPH oxidase, has been shown to be functionally deficient in patients with chronic granulomatous disease (4).

In recent years prostaglandins of the E series and PGI_2 have been shown to inhibit acute inflammatory reactions *in vivo* (5,6) as well as inhibit neutrophil functional responses to a variety of soluble and particulate stimuli *in vitro* (7-10). The inhibition of neutrophil function by PGs has been associated with

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ABBREVIATIONS:
PMN's: Polymorphonuclear leukocytes
PGs: Prostaglandins
cAMP: cyclic adenosine monophosphate
FMLP:formyl-metionyl-leucyl-phenylalanine
15-M-PGE_1:15(S)-15-methyl-prostaglandin E_1
HBSS: Hank's balanced salt solution
O_2^-: superoxide anion
PMA: phorbol myristate acetate

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their ability to increase intracellular cyclic adenosine monophosphate levels. In an effort to better understand the mechanism(s) by which PGs modulate acute inflammatory reactions and activation of the neutrophil NADPH oxidase system we have examined the effects of 15-M-PGE_1 and PGI_2 on the production of O_2^- by human neutrophils after stimulation with three soluble stimuli; the chemotactic formyl peptide formyl-methionyl-leucyl-phenylalanine, phorbol myristate acetate (PMA) and arachidonic acid (a cis-polyunsaturated fatty acid). Each of these stimuli have been shown to induce superoxide production by human neutrophils (11-13).

MATERIALS AND METHODS

Cells: Human peripheral neutrophils were obtained from normal volunteers and purified by Ficol-Hypaque gradient centrifugation followed by hypotinic lysis. Cell preparations contained greater than 90% neutrophils and cell viability was greater than 90% as determined by trypan blue exclusion. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Arachidonic acid was purchased from Nu-Chek Prep (Elysian, MN) and diluted in ethanol at a concentration of 10^{-1}M and stored under nitrogen. 15-M-PGE_1 and PGI_2 were a generous gift of Dr. John Pike (Upjohn Chemical Co., Kalamazoo, MI). Stock solutions of FMLP were prepared at a concentration of 10^{-2}M in dimethylsulfoxide.

Assays: Superoxide anion production by human neutrophils was determined by measuring the superoxide dismutase inhibitable reduction of ferricytochrome C to ferrocytochrome C at 550 nM. Briefly human neutrophils suspended at a concentration between 1-5 x 10^6 cells/ml in Hanks balanced salt solution, (HBSS), pH 7.4, containing 0.1 mM ferricytochrome C were preincubated at 37° x 5 min. in the presence or absence of the test reagents (prostaglandins, etc.). The reactions were initiated by the addition of the specific stimulus at a final volume of 0.7 mls and incubated at 37°C x 10 min. The reactions were terminated by the addition of 25 µl superoxide dismutase (1 mg/ml) and 275 µl of HBSS. The cells were centrifuged and the optical adsorbance of the supernate determined at 550 nm. The amount of O_2^- produced was calculated from the difference in adsorbance between samples of cells who received SOD prior to activation by stimulus and those receiving SOD after activation. The difference was divided by the extinction coefficient for the change between ferricytochrome C and ferrocytochrome C to determine nmoles O_2^- produced per given quantity of cells. The data is expressed as mean values from triplicate samples ± S.E.M. In those reactions in which FMLP was the stimulus the cells were also treated with cytochalasin B (5 µg/ml) after preincubation with the specific inhibitor but prior to addition of FMLP. In those experiments in which the rate of O_2^- production was determined the reduction of ferricytochrome C was continuously monitored in a Cary 210 double beam spectrophotometer.

RESULTS DISCUSSION

Preincubation of the human neutrophils with either 15-M-PGE_1 or PGI_2 inhibited f Met-Leu-Phe induced O_2^- production in a dose dependent manner (Table 1 and 2). At a concentration of 10 µM 15-M-PGE_1 there was 59.8% inhibition of FMLP (10^{-7}M) induced O_2^- production. In contrast $PGF_{2\alpha}$ showed no signficant

Table 1: Inhibition of FMLP induced neutrophil O_2^- production by prostaglandins

	O_2^- nmoles/2x10^6 cells*	% Inhibition	p value**
	Experiment #1		
FMLP (10^{-7}M)	18.9 ± 1.4	-	-
+ 15-M-PGE$_1$ ($4x10^{-5}$M)	3.1 ± 0.6	83.6	< .01
+ 15-M-PGE$_1$ (10^{-5}M)	7.6 ± 0.5	59.8	< .01
+ 15-M-PGE$_1$ ($2.5x10^{-6}$M)	11.8 ± 0.3	37.6	< .01
+ 15-M-PGE$_1$ ($6.25x10^{-7}$M)	14.6 ± 1.2	22.7	< .02
	Experiment #2		
FMLP (10^{-7}M)	18.8 ± 0.5	-	-
FMLP (10^{-7}M) + 15-M-PGE$_1$ (10^{-5}M)	13.1 ± 0.4	30.3	< .01
***FMLP (10^{-7}M) +15-M-PGE$_1$ (10^{-5}M) (washed 4°Cx2)	19.3 ± 0.3	-2.6	NS

* data represent mean values ± standard error of the mean (S.E.M.).

** p values were calculated using students t-test (two-tailed) analysis

*** cells were pre-treated with 15-M-PGE$_1$ for 5 minutes at 37°C, washed in HBSS x2 at 4°C, incubated at 37°C x 10 minutes, then stimulated with FMLP.

inhibition of FMLP induced superoxide production by human PMNs. Other agents known to increase intracellular cyclic AMP including isoproterenol, theophylline, and dibuturyl cyclic adenosine monophosphate also inhibit FMLP induced O_2^- production (Table 2). The inhibition of FMLP induced O_2^- production by 15-M-PGE$_1$ was reversible (Table 1). Neutrophils preincubated with 15-M-PGE$_1$ (10^{-5} M) were washed two times in HBSS (4°C) and after a 10 min incubation at 37° produced 19.3 nmoles O_2^-. This is similar to control cells that were not treated with 15-M-PGE$_1$. This is in contrast to the 30.3% inhibition of O_2^- production observed with cells that were exposed to 15-M-PGE$_1$ throughout the treatment procedure. In additional experiments we observed that no preincubation time was required for the inhibition of FMLP induced O_2^- production by PGI$_2$

Table 2: Effect of prostaglandins, isoproterenol, and dibuturyl cyclic AMP on FMLP and PMA induced O_2^- production

	O_2^- nmole/2x10^6 cells*	% Inhibition	p value**
Experiment #1			
PMA (20 mg/ml)	22.2 ± 1.2		
+ 15-M-PGE_1 (30 μM)	21.6 ± 0.6	2.7	-
+ Isoproterenol (5x10^{-6}M)	21.6 ± 0.5	2.7	-
+ Isoproterenol + theophylline (5x10^{-4}M)	21.2 ± 1.0	4.5	-
FMLP (10^{-7}M)	23.6 ± 0.6	-	-
+ 15-M-PGE_1 (30 μM)	6.3 ± 0.3	71.6	< .01
+ Isoproterenol (5x10^{-6}M)	15.2 ± 0.2	31.5	< .02
+ Isoproterenol + theophylline (5x10^{-4}M)	2.8 ± 0.9	87.3	< .01
Experiment #2			
PMA (20 ng/ml)	29.3 ± 0.4	-	-
+ 15-M-PGE_1 (30μM)	31.4 ± 0.1	-7.1	-
+ PGI_2 (30μM)	32.0 ± 0.3	-9.9	-
+ DcAMP (10^{-3}M)	29.0 ± 0.3	1.0	-
FMLP (10^{-7}M)	23.4 ± 1.4	-	-
+ 15-M-PGE_1 (30μM)	6.5 ± 0.7	72.0	< .02
+ PGI_2 (30μM)	11.6 ± 0.6	47.7	< .01
+ DcAMP (10^{-3}M)	6.9 ± 0.6	70.5	< .01

* Data represent mean values ± S.E.M.

** p values were calculated as described in Table 1.

and 15-M-PGE_1. However if 15-M-PGE_1 or PGI_2 were added 10 seconds or later after FMLP exposure there was no inhibition of O_2^- production (data not shown).

In contrast to the inhibitory effect of 15-M-PGE_1 and PGI_2 on FMLP induced O_2^- production, neither prostaglandins significantly inhibited PMA (Table 2) or arachidonic acid induced O_2^- (Table 3). This was not a dose related effect since there was no alteration in the dose response curves for the production of O_2^- by

Table 3: Effect of 15-M-PGE_1 on arachidonic acid induced O_2^- production

	O_2^- nmoles/4 x 10^5	
Arachidonic acid concentration	Control	+ 15-M-PGE_1 (100μM)
	Experiment #1	
50μM	6.0 ± 0.3	6.4 ± 0.4
25μM	2.5 ± 0.2	2.2 ± 0.4
12.5μM	1.2 ± 0.2	1.1 ± 0.2
100μM	10.5 ± 0.3	10.7 ± 0.2
50μM	6.0 ± 0.3	5.6 ± 0.8

* Data represent mean values ± S.E.M.

either PMA or arachidonic acid in the presence of 15-M-PGE_1. In addition, neither prostaglandin altered the maximum rate of production of O_2^- by either stimulus.

These observations are consistent with previous reports that have demonstrated inhibition of neutrophil chemotaxis (7), aggregation (8), lysosomal enzyme release (9), and particulate stimuli induced O_2^- production (10) by prostaglandins of the E series and PGI_2. In addition the data presented here suggest that the NADPH oxidase system of neutrophils can be stimulated to produce O_2^- by at least two mechanisms; one regulated by prostaglandins and cAMP and the second independent of prostaglandin and cAMP modulation. Since FMLP stimulated O_2^- production was inhibited by PGs at concentrations previously shown to increase intracellular cyclic AMP levels (7-10), the data are in keeping with the hypothesis that PG modulation of the biologic response of neutrophils is a result of increased intracellular cyclic AMP levels. However one cannot exclude the possibilities that PGEs and PGI_2 may have alternative effects on neutrophil cell membranes that may result in the modulation of the activation of the NADPH oxidase system by specific stimuli.

Several reports have indicated that sodium, potassium, and calcium ion fluxes as well as changes in transmembrane potential associated with formyl

peptide stimulation of neutrophil precede superoxide production and lysosomal enzyme release (14-16). Data presented here demonstrates an inability of PGs to modulate FMLP induced O_2^- production when the prostaglandins are added 10 seconds or later after FMLP stimulation. This data suggest that prostaglandins modulate neutrophil function by altering the early biochemical response following stimulation possibly by altering the fluxes of monovalent and/or divalent cations across the cell membrane. It has been suggested that the inhibition of platelet activation by PGI_2 is a result of the activation of a calcium-ATPase by cyclic AMP with sequestration of calcium in the dense tubular system (17). However, the demonstration of similar mechanisms in the neutrophil require additional study.

A specific receptor for phorbol esters on the human neutrophil has been described (18-19). A recent report characterized the phorbol diester receptor in rat brain as having protein kinase C activity (20). Protein kinase C is a calcium activated phospholipid dependent protein kinase which is activated by unsaturated diacylglyerol and can also be activated by PMA causing phosphorylation of a specific 40,000 dalton protein in the human platelet (21). Although a PMA activated protein kinase C in the human neutrophil has not been comparably characterized, the data is consistent with the hypothesis that prostaglandins modulate neutrophil stimulation at a step prior to activation of a PMA activatable step, possibly protein kinase C. However PMA may have other effects on the neutrophil cell membrane that would account for the lack of inhibition of PMA O_2^- production by PGs. Since PMA has been shown to alter the fluidity and hydration of phospholipid bilayers in vitro (22) it is possible that PMA may partition into the neutrophil cell membrane independent of receptor binding and alter the biophysical characteristics of the cell membrane resulting in activation of the NADPH oxidase system. In conjunction with this hypothesis a recent report has demonstrated that arachidonic acid and other cis-polyunsaturated fatty acids will induce superoxide production by human neutrophils in the presence of inhibitors of both the cyclooxygenase and lipoxygenase pathways (13). Although the mechanism by which arachidonic acid and other cis-polyun-

saturated fatty acids stimulate superoxide production is not known our data indicate that whatever this mechanism may be it is not modulated by PGE_1 or PGI_2.

In summary, the data presented here indicate that 15-M-PGE_1 and PGI_2 will inhibit formyl peptide induced superoxide production by human neutrophils in a rapid reversible manner. However the lack of effect of 15-M-PGE_1 and PGI_2 on PMA and arachidonic acid induced superoxide production suggest that alternative mechanisms are present that will activate the NADPH oxidase system of the human neutrophil cell membrane independent of prostaglandin modulation.

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