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THE EFFECT OF SIX PROSTAGLANDINS, PROSTACYCLIN AND ILOPROST ON GENERATION OF SUPEROXIDE ANIONS BY HUMAN POLYMORPHONUCLEAR LEUKOCYTES STIMULATED BY ZYMOSAN OR FORMYL-METHIONYL-LEUCYL-PHENYLALANINE

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Abstract—Prostaglandins (PG) E_2,E_1,6-keto-E_1 and D_2 at concentrations of 0.15–0.80 μM inhibited by 25% the generation of superoxide anions (O_2^-) in human polymorphonuclear leukocytes (PMNs) stimulated with formyl-methionyl-leucyl-phenylalanine (FMLP). The potency of that inhibition by either PGD_2 or PGE_1 was the same when zymosan was used as a stimulator whereas PGE_2 and 6-keto-PGE_1 were by 13 and 21 times less potent inhibitors of O_2^-) in zymosan-stimulated as compared to FMLP-activated PMNs. $PGF_{2\alpha}$ inhibited the generation of O_2^- by activated PMNs only when used at the highest concentration studied (30 μM). Prostacyclin, 6-keto-$PGF_{1\alpha}$ and Iloprost (a carbacyclin analogue of prostacyclin) at concentrations up to 30 μM showed no significant inhibition of O_2^- in human PMNs stimulated either with FMLP or with zymosan. It is concluded that PGD_2 and PGEs use a common basic mechanism for inhibition of the generation of O_2^- by PMNs activated with FMLP or zymosan. PGD_2 is most generously furnished with these properties. In addition to this basic mechanism PGE_2 and 6-keto-PGE_1 abrogate the FMLP-induced response by occupation of formyl peptide receptor of PMNs. It is hypothesised that inhibition of the generation of O_2^- in PMNs and, possibly, in other cells by PGD_2, PGE_2 and by products of prostacyclin biotransformation might be responsible for their cytoprotective action in myocardial infarction, stroke, liver damage and peripheral vascular disease.

Two basic biochemical functions of polymorphonuclear leukocytes (PMNs) are the release of enzymes from granules and the generation of oxygen free radicals by the membrane-bound NADPH-dependent oxidase [1, 2]. These functions of PMNs are triggered by exogenous factors such as bacterial formylated peptides [3], opsonized particles or immune complexes [4], and modulated by endogenous products of cyclo-oxygenation [5–9] and lipoxygenation [8, 10, 11] or arachidonic acid.

PMNs are known to invade ischaemic myocardium [12, 13]. PMNs-derived hydrolytic enzymes [13], leukotrienes [14] and oxygen free radicals [14, 15] are supposed to play an important role in the post-ischaemic reperfusion damage. Therefore, new facts on the modulation by prostaglandins of the univalent reduction of oxygen in activated PMNs might be of importance for understanding of mechanisms of cytoprotection.

MATERIALS AND METHODS

Preparation of opsonized zymosan. A suspension of 50 mg of zymosan in 5 ml of saline was boiled for 1 hr and centrifuged at 1300 *g* for 5 min, the sediment resuspended in 1 ml of saline, 3.5 ml of pooled human serum added and incubated with shaking for 30 min at 37°, centrifuged at 1300 *g* for 10 min and the sediment resuspended in 1 ml of PBS. This final suspension was used *ex tempore* for stimulation of PMNs.

Preparation of prostanoid solutions. Prostanoids were dissolved in ethanol (1 mg/10 μl) and 990 μl of PBS was added. This stock solution of prostanoids was diluted as required with a 1% solution of ethanol in PBS. Prostanoids or 1% ethanol in PBS (either in volume of 20 μl) were added to 2 ml samples of PMNs and thus both experimental and control samples contained 0.01% ethanol but differed by presence or absence of prostanoids. Because of its chemical instability sodium salt prostacyclin (Floalan, Wellcome Research Labs) was prepared as the above solution a few seconds before its instillation to a suspension of PMNs.

Isolation of PMNs. PMNs were isolated from fresh venous blood (3.8% sodium citrate : blood, 2 : 8 v/v) withdrawn by venipuncture from healthy volunteers. A mixture of citrated blood and of 6% dextran in saline (4 : 1 v/v) was left for 60–70 min at 37° until erythrocytes settled down. Further manipulations were carried out at 4°. A "foamy coat" containing PMNs was centrifuged at 1000 *g* for 10 min, washed twice with saline (each time centrifuged at 500 *g* for 5 min) and the hypotonic lysis of erythrocytes remaining in the sediment was repeated twice, in the following way. The sediment of PMNs was treated with 10 ml of 0.2% NaCl solution for a period of 20 sec and then supplemented with 10 ml of 1.61% NaCl solution in order to return to isotonic conditions at which the suspension of PMNs was centrifuged at 800 *g* for 8 min. The sediment containing about 90% of PMNs was washed twice with saline and centrifuged, then resuspended in PBS to yield 2.5–

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3.5×10^6 cells/ml. Viability was determined by Trypan blue exclusion and cells were more than 90% viable at all time period tested.

Design of experiments. Concentration of superoxide anions (O_2^-) in a suspension of zymosan- or FMLP-stimulated PMNs was assayed by spectrophotometric evaluation of the reduction of ferrocytochrome C to ferricytochrome C [16]. All samples comprised $2.5–3.5 \times 10^6$ PMNs in 1 ml of PBS and blank samples additionally contained superoxide dismutase (SOD, 25 μg/ml). The experimental samples contained prostaglandins at final concentrations of 0.03–20 μM and the control samples their solvent (20 μl). The samples were incubated with shaking for 5 min at 37° and then supplemented with ferrocytochrome C in 0.9 ml of saline to yield a final concentration of 1 μg/ml of ferrocytochrome C in a sample. Following 10 min of preincubation PMNs were stimulated either with opsonized zymosan (final concentration 1 mg/ml) or by FMLP (final concentration 22 μg/ml). Zymosan and FMLP were added in 100 μl of PBS and thus the final volume of each sample was 2 ml. Following the next 10 min of incubation samples were immersed in an ice bath for 5 min, then centrifuged at 600 *g* for 5 min (40°) and 1 ml of the supernatant was diluted with 1 ml of PBS. The extinction of ferricytochrome C was read against PBS at 550 nm using u.v. Spectrophotometer SP 1700 Pye Unicam in triplicates. The cofactor of molar extinction 20,000 M^{-1} cm^{-1} was used. The amount of the produced O_2^- (nmoles/10^6 PMNs/10 min) was calculated using the following equation:

$$\frac{dE}{Q \times d} \times 10^6 \times \frac{V_f}{V_i} \times \frac{1}{L}$$

where dE is an increase in extinction between a sample with and without superoxide dismutase, Q is the cofactor of molar extinction, d is the thickness of the cuvette in cm, V_f and V_i are the final and initial volumes and L is a number of PMNs in a sample multiplied by 10^{-6}.

Reagents. Cytochrome C type VI from horse heart (Sigma, St Louis, MO), superoxide dismutase from bovine erythrocytes (Sigma), zymosan A from Saccharomyces Cerevisiae (Sigma), *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, chemotactic peptide) (Sigma), prostaglandins E_1, E_2, 6-keto-E_1, 6-keto-$F_{1\alpha}$, $F_{2\alpha}$ (Sigma), D_2 (Upjohn Co and Sigma), prostacyclin (PGI_2 sodium salt, crystalline or lyofilized Floalan, Wellcome Research Laboratories), Iloprost (Schering AG), Dextran T 500 (Fine Farmacia). Phosphate buffered saline (PBS) according to Dulbecco was contained in 1000 ml of bidistilled water (g): 8.09 NaCl; 0.29 KCl; 0.29 KH_2PO_4; 1.159 anhydrous Na_2HPO_4; 0.019 $CaCl_2$; 0.018 $MgCl_2 \cdot 6H_2O$; 1.0 glucose—and had pH 7.4.

RESULTS

Human PMNs when activated with FMLP or zymosan produced 110 ± 7 nmoles $O_2^-/10^6$ cells/10 min (mean ± SE, N = 37) and 119 ± 9 nmoles $O_2^-/10^6$ cells/10 min (mean ± SE, N = 31), respectively. Prostaglandin, its product of hydrolysis 6-keto-$PGE_{1\alpha}$ and its carbacyclin analogue Iloprost [17] at a range of concentration from 0.3 to 30 μM inhibited neither zymosan-stimulated nor FMLP-activated generation of O_2^- by human PMNs. $PGF_{2\alpha}$ only at a highest studied concentration of 30 μM had a significant inhibitory action (Fig. 1). Analysis of variance showed linearity of regression for none of the above four prostaglanoids whereas the linearity of inhibitory responses to PGE_1, PGE_2, 6-keto-PGE_1 and PGD_2 was highly significant ($P < 0.01$). Two prostaglandins of the E series, i.e. PGE_2 and 6-keto-PGE_1 were significantly ($P < 0.05$) more potent inhibitors of FMLP- than of zymosan-induced generation of O_2^- with the ratio of potencies of 21 and 13, respectively (Table 1). In contrast with PGE_2 and 6-keto-PGE_1 the inhibitory potency of either PGD_2 or PGE_1 against both stimuli (i.e. FMLP or zymosan) did not differ significantly from each other (Table 1). These four prostanoids whereas the linearity of inhibi-induced generation of O_2^- in a concentration-dependent manner starting from 0.3 μM for PGE_1 and 6-keto-PGE_1 and from 0.03 μM for PGE_2 and PGD_2 (Fig. 1). The most potent inhibitor of generation of O_2^- was PGD_2 with IC_{25} of 0.15 μM and 0.21 μM for the induction by FMLP and zymosan, respectively (Table 1). In FMLP-activated PMNs all four anti-O_2^- active prostaglandins had similar inhibitory potencies (IC_{25} from 0.15 to 0.8 μM) and similar slopes of regression lines (b from 26.3 to 34.2) (Table 1). Major differences among anti-O_2^- potencies of these prostaglandins appeared in zymosan-stimulated PMNs. IC_{25} values from 0.21 to 10.56 μM and slopes of regression lines (b) from 10.4 to 30.1 (Table 1).

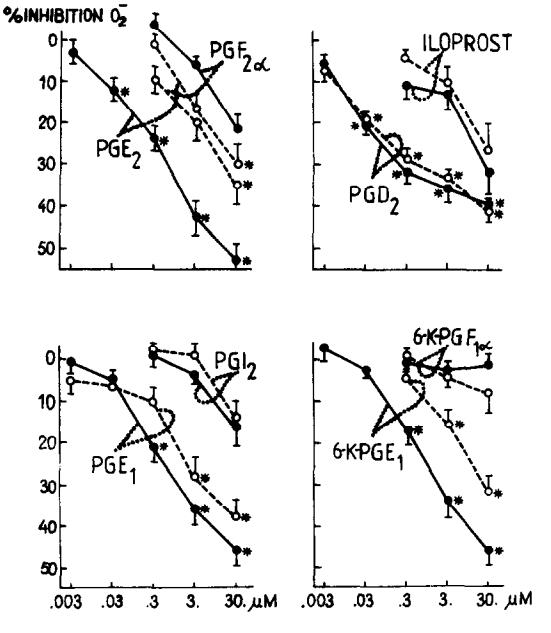

Fig. 1. Inhibition by eight prostanoids of the generation of O_2^- in FMLP-stimulated (●—●) and zymosan-stimulated (○···○) human PMNs. Ordinate—percent of inhibition; abscissa—concentrations of prostanoids in log scale (0.03–30 μM). Each point represents mean of 9–12 experiments, vertical bars, SE. Asterisks denote significant inhibition ($P < 0.05$).

Table 1. Inhibitory effect of four prostaglandins on the generation of superoxide anion (O_2^-) by human polymorphonuclear leukocytes (PMNs) stimulated with formyl-methionyl-leucyl-phenyl-alanine (FMLP) or zymosan

Inhibitor	Stimulator	N	a	b	IC_{25} (μM)	95% Confidence limits(μM)	P
PGE_1	FMLP	31	12.3	28.5	0.52	0.21– 1.31	>0.05
	Zymosan	40	9.4	22.4	1.90	0.55– 8.92	
PGE_2	FMLP	48	12.8	34.2	0.19	0.09– 0.36	<0.05
	Zymosan	26	9.2	19.5	4.00	0.53–68.94	
6-keto-PGE_1	FMLP	31	13.2	26.3	0.80	0.35– 1.88	<0.05
	Zymosan	26	14.2	10.4	10.56	4.36–34.42	
PGD_2	FMLP	43	8.3	31.7	0.15	0.04– 0.42	>0.1
	Zymosan	15	7.6	30.1	0.21	0.05– 0.59	

Regression equations ($y = ax + b$) were constructed [46] for percent inhibition (y) of generation of O_2^- by FMLP- or zymosan-activated human PMNs by four prostaglandins at concentrations of $x = \log[\mu M]$ in N experiments. Linearity of each of eight regression lines was proven by analysis of variance ($P < 0.01$). IC_{25} values with its 95% confidence limits were calculated and logarithm relative potency was used to find probability (P) for a significant distance within a pair of FMLP/zymosan regression lines for each prostaglandin [46].

DISCUSSION

Human PMNs when activated with FMLP, opsonized zymosan, calcium ionophore A 23187, phorbol myristate acetate (PMA) or arachidonic acid generate oxygen free radicals and secrete the content of their granules [3, 5–7, 16, 18–20]. Superoxide anions (O_2^-) are derived from univalent reduction of molecular oxygen by membrane-bound NADPH-oxidase [1, 2] whereas hydroxyl radicals ($OH\cdot$) may also arise in the course of biosynthesis of prostanoids [21]. Prostaglandins of the E series (PGEs) [6, 7, 9, 20] and PGD_2 [20] have been shown to inhibit the outburst of production of O_2^- induced by FMLP [6, 19, 20] or by zymosan [7] but not by PMA, arachidonic acid A 23187, although PGEs do inhibit the degranulation of PMNs induced by all five agents [5–7, 19] and an increase in c-AMP by PGEs and PGD_2 occurs in PMA-treated PMNs to the same extent as in FMLP-stimulated PMNs [20]. There exists a clear and direct evidence [1, 7, 21] that the production of O_2^- and the secretion of lysosomal by activated PMNs are concurrent but dissociable processes which are subsequent to earlier ligand–receptor interaction on the PMNs plasmalemma. Moreover, there is little doubt that prostaglandins do use different routes for the suppression of the generation of O_2^- and for the inhibition of lysosomal enzyme release [6, 7, 19]. The fact that in our hands and in some [7] but not all [20] other hands PGs suppressed the generation of superoxide anions in PMNs induced by both a water soluble stimulus—FMLP and a particulate stimulus—opsonized zymosan may indicate that these two stimuli so different in their nature trigger a common PG-sensitive pathway which is different from that triggered by PMA, A 23187 or arachidonic acid.

We were able to confirm previous reports [6, 7, 19] concerning the inhibitory effects of PGE_2 and PGE_1 on the production of O_2^- by FMLP- and zymosan-activated PMNs and a poor effectiveness of $PGF_{2\alpha}$ in this respect. However, contrary to these reports [6, 7] we were unable to demonstrate a significant inhibitory effect of prostacyclin (up to 30 μM) on the generation of O_2^- by activated PMNs. Neither a stable product of prostacyclin hydrolysis, 6-keto-$PGE_{1\alpha}$, nor a synthetic prostacyclin analogue, Iloprost, were efficient O_2^- inhibitors.

Recently it has been reported [9] that prostacyclin and Iloprost at concentrations of 150–3000 μM inhibit the production of O_2^- by zymosan-activated canine PMNs. This effect of prostacyclin and Iloprost does occur at concentrations of 4–5 orders of magnitude (!) higher than a concentration of PGD_2 reported here to render a significant inhibitory effect in human PMNs. Submicromolar concentrations of PGD_2 at which this prostaglandin is effective as anti-O_2^--agent in human PMNs ought to be remembered as a reference point for a similar action of any prostanoid.

FMLP-activated human and rat PMNs when added to cultured endothelial cells evoke the biosynthesis of prostacyclin [22]. According to our present data this endothelial prostaglandin is not capable of deactivating PMNs unless converted to 6-keto-PGE_1 [23] or perhaps to another biologically active metabolite [24]. Presently, for the first time we have shown that a stable and biologically active metabolite of prostacyclin-6-keto-PGE_1 [23] is a potent inhibitor of O_2^- from activated human PMNs. Among prostaglandins studied PGD_2 was not only the most potent inhibitor of O_2^- but also it differed qualitatively from its isomer PGE_2. Thus, PGE_2 was a 21 times less potent inhibitor of zymosan-induced than of FMLP-induced generation of O_2^-, whereas PGD_2 showed similar inhibitory potencies toward zymosan- and FMLP-activated PMNs. In other words, PGE_2 was a preferential antagonist of FMLP-induced generation of O_2^- while PGD_2 was not. PGE_2 showed also a PGD_2-like component of "non-FMLP-receptor-mediated" inhibition, although it was 20 times less potent than PGD_2 in this respect. Unfortunately in our assay of superoxide anions we did not use cytachalasin B, which could help to separate differences between the modes of activation of PMNs

by FMLP and zymosan and the routes of inhibition of PGE_2 and PGD_2.

Perhaps a difference between the mode of action of PGE_2 and PGD_2 is that the former one—but not the latter—combines with the formyl peptide receptors on PMNs [3, 6, 25] and thus hinders an increase in inositol triphosphate and diacylglycerol, followed by mobilisation of intracellular calcium [26, 27], activation of protein C kinase [28] and, finally, by an outburst of the generation of O_2^- [3, 27]. A decreased receptor occupancy by FMLP following exposure to PGEs has been considered as a possible mechanism of their inhibitory action on the generation of superoxide anions [7] or such a possibility has been denied [20]. We propose that PGE_2 obliterates this FMLP-receptor-mediated response in addition to having a common with PGD_2 mechanism of anti-O_2^- action in PMNs. By analysis of variance we have shown that 6-keto-PGE_1—but not PGE_1—shares with PGE_2 this receptor-mediated O_2^--suppressive action.

Common mechanisms of anti-O_2^- action of PGD_2 and PGEs may comprise the inhibition of lipoxygenase activity in PMNs as it has been demonstrated for PGEs [8, 29]. In consequence, the generation of products of 5-lipoxygenase, i.e. leukotrienes including leukotriene B_4 (LTB_4) and of products of 15-lipoxygenase, i.e. lipoxins including lipoxin A (LXA) would be abrogated. LTB_4 and LXA both are synthetised by phagocytising and receptor-activated PMNs and they induce the generation of O_2^- and the secretion of granular enzymes [10, 11]. Hence the logistics for the equipotent action of PGD_2 in suppressing zymosan-stimulated and FMLP-activated generation of O_2^- in PMNs.

Another possibility is that PGD_2 acts as an antagonist of LXA. Unlike LTB_4—a potent degranulating agent—LXA is an ideal candidate for a selective stimulator of the membrane-bound NADPH-oxidase in activated PMNs [10, 11]. There are many examples known of antagonistic relations which exist between pairs of arachidonic acid metabolites, to mention only opposite effects of PGE_2 in bronchi or of PGI_2 and TXA_2 in platelets. PGD_2 and LXA may constitute such an antagonistic pair which affects the process of the generation of O_2^- in PMNs.

Apart from this enzyme-mediated or receptor-mediated inhibition of the generation of O_2^- in PMNs there still exists a possibility that PGD_2 and PGEs act as subunits in a biological system which scavenges O_2^-. Recently, it has been hypothesised [30] that contrary to prostacyclin the products of its biotransformation such as 6-keto-PGE_1 [23] and others [24] exert the cytoprotective action owing to their ubiquinone-like properties in ischaemic mitochondria [31]. The cytoprotection by prostaglandins or prostacyclin in stomach [32], liver, kidney [33], pancreas [34], brain [35] and heart [9] might relay on the activation of a cellular system which would scavenge O_2^-—perhaps not only in PMNs. Could it be that this activation is a common denominator for the beneficial effects of PGE_1 and prostacyclin (or rather products of its biotransformation [23, 24]) in patients with peripheral vascular disease [36–38], myocardial infarction [39], certain types of angina at rest [40] and stroke?

A high anti-O_2^- potency of PGD_2 in leukocytes might be of special importance for the cardioprotection. Mast cells (efficient producers of PGD_2) tend to accumulate in adventitia of coronary arteries of patients with atherosclerosis [41] and angina [42]. The IgE-reduced release of PGD_2 from cardiac mast cells has been proposed to influence the course of myocardial infarction in man [43]. "Endogenous nitroglycerine"—EDRF is destroyed by O_2^- [44]. Vasoprotective action of prostaglandins is likely to be associated with scavenging of O_2^- from activated PMNs which are rolling over the endothelium.

In summary, we have demonstrated that prostaglandins E_1, 6-keto-E_1, E_2 and D_2 at submicromolar concentrations inhibit the appearance of SOD-sensitive, cytochrome C-reducing oxygen species which are generated by FMLP-activated human PMNs. In zymosan-activated PMNs the same high anti-O_2^- potencies have been found for PGD_2 and PGE_1, but not for PGE_2 and 6-keto-PGE_1. Prostacyclin, 6-keto-$PGF_{1\alpha}$ and Iloprost at concentrations up to 30 μM hardly inhibit the generation of O_2^- by activated human PMNs. It is proposed that PGD_2, PGE_2 and metabolites of prostacyclin such as 6-keto-PGE_1 may exert their cytoprotective action through a suppression of the generation of O_2^- in PMNs and, possibly, in other cells.

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