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INHIBITION OF HUMAN POLYMORPHONUCLEAR LEUKOCYTE RESPIRATORY BURST ACTIVITY AND AGGREGATION BY 6-KETOCHOLESTANOL

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6-ketocholestanol, a naturally occurring oxygenated sterol, when incubated with human neutrophils (PMN), can inhibit superoxide and hydrogen peroxide generation in a dose-dependent fashion. This is accompanied by inhibition of stimulated PMN aggregation without alteration in cellular viability. This inhibitory effect is not affected by washing of the cells, and cannot be blocked by the addition of free cholesterol to the medium. These data are consistent with prior observations which showed an inhibitory effect on PMN chemotaxis by certain oxygenated sterol compounds, and support the hypothesis that certain oxygenated sterols can affect a variety of human PMN functions by a mechanism that may involve perturbation of the plasma membrane.

KEY WORDS: sterols, neutrophils, 6-ketocholestanol.

INTRODUCTION

Oxygenated sterol compounds (OSC), potent inhibitors of sterol synthesis in a variety of mammalian cells,[1] also regulate certain membrane dependent functions in polymorphonuclear leukocytes.[2] We hypothesized that OSC insertion into plasma membranes may explain some of these effects.[2,3] Certain OSC inhibit chemotaxis of human polymorphonuclear leukocytes in a dose- and time-dependent manner without significantly altering random movement.[2,3]

In our current experiments we describe the effects of 6-ketocholestanol (6-KC) on two membrane dependent functions in human PMN, respiratory burst activity and aggregation. We found that 6-KC inhibits superoxide (O_2-) and hydrogen peroxide (H_2O_2) production, as well as PMN aggregation in a dose-dependent fashion. We believe these data support the hypothesis that certain OSC affect a variety of human PMN functions, perhaps by perturbation of the plasma membrane.

MATERIALS AND METHODS

OSC

Cholesterol and 6-ketocholestanol were obtained from steraloids (Wilton, New

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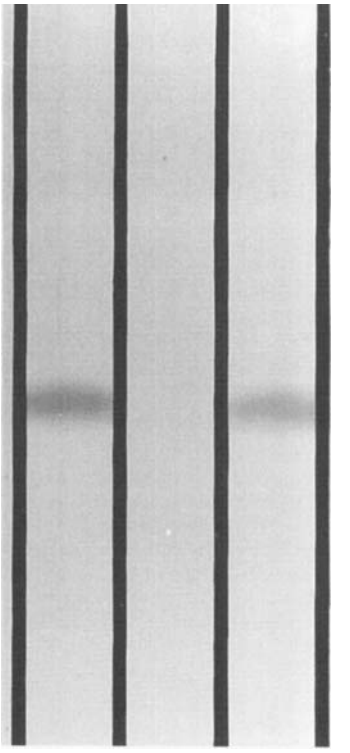

FIGURE 1 Thin layer chromatography demonstrates one band (shown in two lanes) representing 6-KC. 10 ug of 6KC was placed in the lanes and the plates developed as described in Materials and Methods.

Hampshire). Purity of 6-Ketocholestanol was determined by thin layer chromatography (Figure 1) and one band was isolated using LMP-KDF Whatman plates and solvent containing 90% Ethyl acetate and 10% Hexane. 10 ug of 6KC was placed in each lane and the bands were developed with a solution containing 5% acetic acid, 5% H_2SO_4. Phorbol myristate acetate (PMA) was purchased from Consolidated Midland Corporation, Forrester, New York, and was stored at $-4°C$ at a stock concentration of 10 mg/ml in dimethylsulphoxide (DMSO) and used in a final concentration of 10 ng/ml for O_2 and H_2O_2 determination. N-formyl-leucyl-methionyl-phenylalanine (FMLP) was purchased from Sigma Chemicals, St. Louis, Missouri, and stored at a stock concentration of 10^{-6} M and used in a final concentration of 10^{-7} M. Serum treated zymosan was washed in PBS stored at 25 mg/ml at 20°C, then incubated with pooled human serum with shaking at 37°C for 30 min.

Isolation of Polymorphonuclear Leukocytes

PMN were isolated as previously described.[4] Briefly, 30–50 cc of blood were drawn into a syringe containing 1 u/ml of heparin and were allowed to sediment with one-half volume of 6.5% dextran and 0.98% sodium chloride. The supernatant was collected and the remaining red cells were lysed by hypotonic lysis. The pellet was then

resuspended in media and layered onto a Ficoll-Hypaque gradient and allowed to centrifuge at 450 × g for 30 minutes. The pellet was collected and contained 95% PMN. The PMN were suspended in Hanks' balanced salt solution (HBSS) plus 0.2% human serum albumin (HBSS-HSA) or HBSS alone. Cell viability was determined by trypan blue dye exclusion and lactic dehydrogenase (LDH) release as previously described.[4]

Superoxide Generation

Superoxide anion (O_2-) generation was determined by the cytochrome C assay as described by Goldstein, *et al.*[5] PMN, suspended in either HBSS or calcium-free HBSS were incubated with varying concentrations of 6-ketocholestanol in ethanol (1%). Superoxide generation was measured as superoxide dismutase inhibitable reduction of cytochrome C measured at 550 nm using a molar extinction coefficient for this change in absorption of 21,000. PMN were stimulated with PMA 10 ng/ml final concentration, or serum treated symosan articles (STZ) 10 mg/ml. Incubations were also carried out with ethanol alone (1%).

Hydrogen Peroxide Determination

Measurement of hydrogen peroxide was determined by peroxidase catalyzed oxidation of phenol red as previously described.[6] Briefly, H_2O_2 standards were prepared and stored at 4°C in an aluminum foil covered flask. Buffered phenol red solution (PRS) contained 140 millimolar sodium chloride, 10 millimolar potassium phosphate buffer (pH 7.0), 5.5 millimolar dextrose, 0.28 millimolar phenol red, and 5.5 u/ml of horseradish peroxidase (HRPO). Phenol red and HRPO were added just prior to the experiment. Experiments were carried out in 1 ml/PRS pre-warmed to 37°C. Cells were stimulated with PMA, then, after 30 minutes incubation, centrifuged, and 10 microliters of 1N sodium hydroxide was added to the supernatant. Samples were read at 610 nm against a blank of 1 ml of PRS to which 10 microliters sodium hydroxide was added. This was compared to an H_2O_2 standard curve performed concomitantly. Results are expressed as nanomoles of H_2O_2 per 2×10^6 cells per 30 minutes.

PMN Aggregation

PMN aggregation was measured by previously described method.[7] Briefly, PMN were incubated with or without 6-KC for 10 minutes in the cuvette of a standard platelet aggregometer. Cells were then stimulated with either FMLP 10^{-7} M final concentration (with cytochalasin B 5 ug/ml for 5 min. prior to FMLP addition) or PMA 10 ng/ml final concentration. Ethanol 1.6% final concentration was the carrier for 6-KC and serves as the control. Change in light transmission was recorded as a deflection of light and was measured from the time of stimulation with known aggregating agents PMA or FMLP and various concentrations of 6-KC.

RESULTS

Effects of 6KC on O_2^- and H_2O_2 Production

6-ketocholestanol at doses ranging from 19 uM to 625 uM significantly inhibited

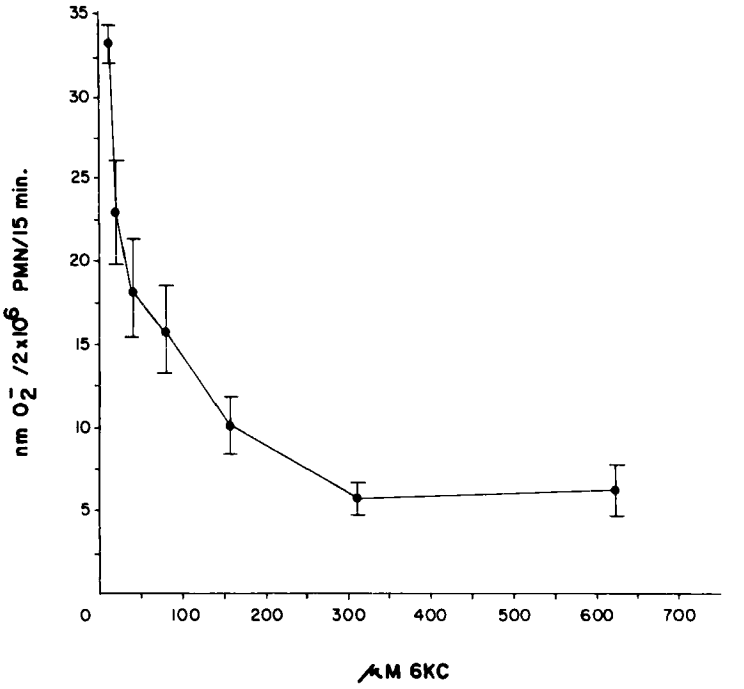

FIGURE 2 Human PMN were incubated with varying concentrations of 6-ketocholestanol ranging from 19 uM to 625 uM for 10 minutes, then stimulated with PMA 10 ng/ml final concentration. O_2 generation is measured and expressed as nanomoles/2 × 10^6 PMN/15′. Incubations with ethanol alone (1%) final concentration did not result in O_2 inhibition. PMA alone 32.0 ± 1.23 (S.E.), n = 31.1% ETOH 31.5 ± 1.24 (S.E.), n = 42.

superoxide production in a dose dependent fashion as shown in Figure 2. Incubation with 1% ethanol alone resulted in no inhibition of O_2^- generation. Similarly, 6-KC inhibited superoxide production when PMN were stimulated with a particulate stimulus, opsonized zymosan particles (STZ) (Table I). We found that there was also inhibition of hydrogen peroxide production by PMN stimulated with PMA when the cells were pre-incubated with 6-KC from 325 uM to 625 uM (Figure 3) but minimal inhibition with 1–2% ethanol alone. Washing of the PMN one time with HBSS after incubation with 6-KC prior to stimulation with PMA had no effect on the ability of 6-KC to inhibit PMN oxidative metabolism (data not shown), and addition of 6-KC

TABLE I
Effects of 6-KC on O_2^- Generation using a particulate (opsonized zymosan particles) stimulus.

Addition to PMN	O_2^- (nm cytochrome C reduced)
1% ETOH	16.2
6-KC 39 uM	3.7
6-KC 160 uM	4.3
6-KC 310 uM	4.3

PMN are incubated with 6KC or ethanol controls, then stimulated with opsonized zymosan particles. O_2^- is then measured as described in materials and methods.

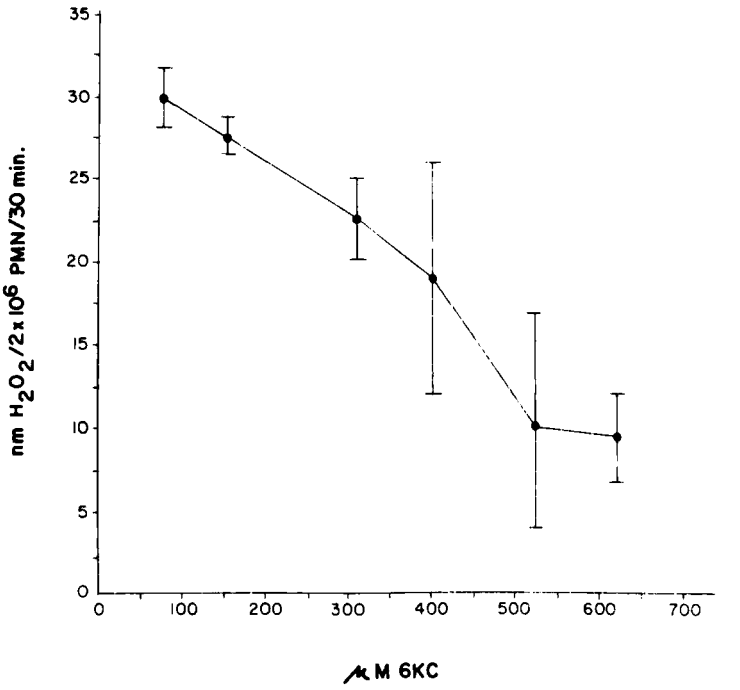

FIGURE 3 6-ketocholestanol pre-incubated with human PMN at varying concentrations and stimulated with PMA 10 ng/ml followed by measurement of hydrogen peroxide generation (expressed as nanomoles/10^6 PMN/30′) as outlined in Materials and Methods. Incubation with ethanol alone resulted in slight inhibition of H_2O_2 generation, PMA alone 33.2 ± 0.83 S.E., n = 3, 1% ETOH 26.0 ± 2.7 SE, n = 6, 2% ETOH 22.7 ± 2.9 S.E., n = 6. There is a dose-dependent inhibition of H_2O_2 generation by 6KC over and above that seen with ETOH alone. The highest concentration of 6KC (625 uM) was dissolved in 2% ETOH.

after agonist stimulation resulted in no inhibition of O_2- generation suggesting that the assay was not being affected. Addition of 6KC to a cell free O_2- generating system (xanthine:xanthine oxidase) resulted in minimal change in the amount of cytochrome C reduced at 12 minutes (1% ETOH = 20.0 nmol, 6KC = 15.3–18.1 nmol), and

TABLE 2

Addition of cholesterol does not block inhibitory effects of 6-ketocholestanol Cholesterol (63 uM) is incubated with PMN for 5′ prior to addition of 6KC for 10′. PMN are then stimulated with PMA 10 ng/ml, and O_2 generation measured as described in Materials and Methods. Results are expressed as nanomoles/ 2 × 10^6 PMN/15′ ± S.E. n refers to the number of experiments.

Addition to PMN	O_2^-	S.E.	(n)
NONE	32.0	±1.23	31
ETOH 1%	31.5	±1.24	42
Cholesterol 63 uM	35.73	±2.04	5
Cholesterol 63 uM + 6KC 313 uM	3.51	±1.73	5
Cholesterol 63 uM + 6KC 156 uM	9.6	±3.10	5
Cholesterol 63 uM + 6KC 78 uM	10.35	±3.23	5
Cholesterol 63 uM + 6KC 39 uM	13.77	±4.12	5

addition of 6KC to the peroxidase assay for H_2O_2 resulted in no inhibition of H_2O_2 detected, and no change in the standard curve (not shown).

We found that preincubation of PMN with cholesterol (63 uM) (Table 2) did not prevent 6KC inhibition of O_2- generation, a finding which differed from our prior observations when chemotactic activity was measured in PMN after OSC exposure.[3]

Effects of 6KC on PMN Aggregation

We examined the effects of varying concentrations of 6KC on PMA and FMLP induced PMN aggregation. We found (Figure 4a) that when FMLP was the agonist there was inhibition of aggregation at 5–25 uM and complete inhibition at 42 uM. There was a consistent dip in the aggregation curve upon exposure to 5.25 uM of 6KC, then recovery (re-aggregation). When PMA was the agonist (Figure 4b) there was a dose dependent inhibition of aggregation at from 23–375 uM. A higher concentration of 6KC was required for inhibition when PMA was the agonist compared to FMLP. Control curves reflect PMN incubated in 1.6% ethanol, the carrier for 6KC in these experiments.

Cell Viability

The effects of 6-KC on cell viability after 30 minutes of incubation at 37° were evaluated using Trypan blue dye exclusion, and release of the cytoplasmic marker

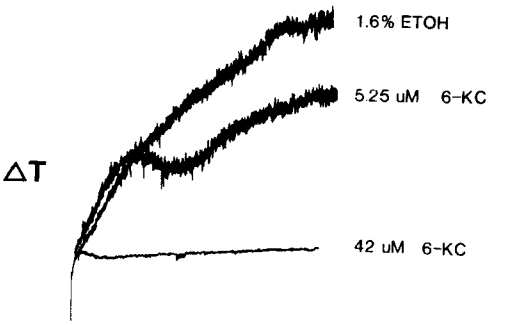

FIGURE 4a Varying concentrations of 6KC (or ethanol 1.6%) are added to cytochalasin-B (5 ug/ml) treated PMN with stirring to a cuvette of a standard platelet aggregometer. FMLP (10^{-7} final concentration) is added and change in light transmission recorded. There is inhibition of aggregation in 6KC treated cells compared to ethanol controls.

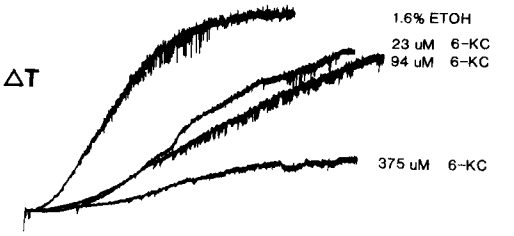

FIGURE 4b PMA (10 ng/ml final concentration) without cytochalasin b is added rather than FMLP and change in light transmission recorded. There is again inhibition of aggregation in 6KC treated cells.

LDH. We found greater than 95% viability in all of the concentrations of 6-KC tested by Trypan Blue and no increased release of LDH at the highest concentrations of 6-KC (in 1–2% ETOH) tested.

DISCUSSION

We have demonstrated that 6-KC, when incubated with human PMN, inhibits respiratory burst activity (O_{2^-} and H_2O_2 production) in a dose-dependent manner. Further, there is also inhibition of PMN aggregation, but no alteration in cellular viability. The inhibitory effects are seen in concentrations starting from 5.25 uM (for aggregation) to 19 uM (for O_{2^-} production) and 325 uM (for H_2O_2 production). Moreover, this inhibition is not affected by washing of the cells and cannot be blocked by addition of free cholesterol to the medium.

The finding that the same concentration of 6-KC (150 uM) results in 70% inhibition of O_2 (Figure 2) but only 16% inhibition of H_2O_2 (Figure 3) cannot easily be reconciled by these experiments. There are several possible explanations which could theoretically account for these observations. Although most of the H_2O_2 generated in our system likely comes from dismutation of O_2^- (spontaneous or catalyzed by SOD), H_2O_2 may also be formed by the direct 2 electron reduction of O_2, a process that may account for the relatively less inhibition of H_2O_2 compared to O_2^-. Further, reduction of H_2O_2 depends upon the haem enzyme catalase or the seleno enzyme glutathione peroxidase. If either 6-KC or its carrier ETOH affects the activity of these enzymes, there may be relatively more H_2O_2 measured than would otherwise be present in this system, thus accounting for the apparent discrepancy in measured H_2O_2 inhibition compared to measured O_2^- inhibition.

Prior studies have shown that OSC may have profound effects on plasma membranes of mammalian cells.[1,2,3] Certain OSC can inhibit sterol synthesis in mammalian cells in tissue culture,[8] and mouse L-cells incubated with OSC show several defects associated with plasma membrane dysfunction.[9,10] We have previously shown that certain OSC, including 6-KC, inhibit chemotaxis in human PMN[2,3] and have suggested that some of the observed effects of OSC are due to PMN membrane perturbation (perhaps by insertion) rather than by inhibition of sterol synthesis. We have here extended these studies, and our findings that respiratory burst activity and aggregation are decreased by pre-incubation with 6-KC is consistent with a membrane mediated effect. That the observed effects are not related to scavenging of generated oxygen radicals is supported by the observation that addition of OSC following PMN stimulation with agonist does not result in reduction of superoxide generation.

Whereas the time required to observe inhibition of sterol synthesis in experiments using mammalian cells was long (2–3 hours),[1] we were able to demonstrate inhibition of aggregation and respiratory burst activity after just 10–15 minutes of pre-incubation, supporting a mechanism of inhibition that involves alteration of plasma membrane components directly rather than alteration of endogenous membrane components which might occur as a consequence of inhibition of sterol synthesis. There are other experimental and theoretical considerations which make it unlikely that our observed effects can be explained as a consequence of inhibition of sterol synthesis, especially since PMN lack certain enzymes necessary for de novo sterol synthesis.[11]

The mechanism of the inhibition of PMN respiratory burst activity has not been

elucidated from our studies. Human PMN, when triggered by certain agonists, can metabolize oxygen to reactive oxidants.[12] These cells take up molecular oxygen at a rapid rate, and utilize a complex NADPH oxidase system which generates superoxide anion, a one electron reduction product.[13,14] Any alteration of the PMN membrane may serve to alter the oxidase system in some way, and our observation that the inhibition of respiratory burst activity by 6-KC is seen both with soluble and particulate agonists suggests a non-specific membrane effect.

The implications of the multiple inhibitory effects of OSC on PMN oxidative metabolism are not yet fully understood, but it appears that there is significant inhibition of two very important components of the inflammatory response. Since certain OSC's have been implicated in the pathogenesis of atherosclerosis[15] and may be toxic to other cells,[16,17] the observation that auto-oxidation products of cholesterol such as 6-KC[18] may regulate PMN oxidative metabolism and aggregation suggests an intrinsic negative feedback inhibitory system at sites of inflammation. The concentrations of 6-KC required to cause O_2^- and H_2O_2 inhibition are high, perhaps 1–2 logs higher than the serum concentrations of other OSC's (21) such as 25 hydroxycholesterol, so we cannot postulate that the effects we have observed account for in vivo antiinflammatory phenomena. However, we do not know if these concentrations are similar to those that might be found in certain foodstuffs (heated butter, etc.) (21), or inflammatory exudates. Further, we cannot correlate serum concentration with biologic effects, since it is likely that 6-KC would partition into membranes where its inhibition effect would be exerted.

Certainly, since only very small concentrations of oxysterol are necessary to regulate HMG-CoA reductase activity in cultured cells and in liver by binding to a cytosolic receptor (22), we doubt that our observations can be explained by a similar mechanism. Indeed, it appears more likely that non specific membrane effects may account for the inhibitory effect we have observed.

The oxidized cholesterol that may occur at inflammatory sites may come from endogenous oxidation of serum cholesterol or may be derived from phagocyte oxidation of cholesterol released by activated platelets (19). Recent studies suggest that inflammatory phagocytes can oxidize serum components (low density lipoproteins, LDL) and make them more toxic to target cells (20). The studies reported herein suggest that a sterol oxidation product (6-KC), which may be carried in oxidized LDL's, may regulate its own production and its contribution to target cell toxicity by interfering with PMN activation, although the concentrations we have employed are possibly higher than one might expect in vivo. The precise mechanism of the effects of 6-KC and its significance regarding a full range of biologic processes, including atherogenesis and inflammation, remain to be explored.

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