FEBS Letters 349 (1994) 13–16

FEBS 14240

Priming effect of hydroxyapatite on the chemiluminescence response in human polymorphonuclear leukocytes

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Received 9 June 1994

Abstract

In order to determine whether hydroxyapatite modulates the response of polymorphonuclear leukocyte (PMN) to oxidative stimuli, human PMNs were incubated with a non-activating concentration (1 or 10 μg/ml) of hydroxyapatite prior to stimulation with *N*-formyl-methionyl-leucyl-phenylalanine (FMLP; 0.1 or 1 μM), phorbol 12-myristate 13-acetate (PMA; 100 pg/ml), sodium fluoride (50 μM), zymosan (1 μg/ml), or the calcium ionophore A23187 (0.1 μM). Chemiluminescence was measured with an automatic microcomputer-controlled luminescence analyzer at 37°C. Hydroxyapatite alone did not stimulate chemiluminescence at concentrations below 10 μg/ml. Levels 300–400% higher than 'stimulus only' controls without preincubation with hydroxyapatite have been recorded. This synergism between hydroxyapatite and subsequent stimuli reveals a new activity of hydroxyapatite and suggests that particulate material may prepare PMNs for an exaggerated inflammatory response to other phlogistic mediators. This is the first report demonstrating PMNs primed with particulate material.

Key words: Particulate material; Crystal; Inflammation; Zymosan

1. Introduction

The deposition of hydroxyapatite microcrystals in tissues is associated with pathological syndromes [1–3]. Clinical and experimental evidence of the fibrogenetic potential of hydroxyapatite particles seems to concur, in part, with data on the ability of the crystals to induce inflammatory cells to generate reactive oxygen metabolites in vitro. While hydroxyapatite crystals have been reported to induce a large array of biological effects [4–6], it is not known what concentration of the crystals would be necessary to induce pathological syndromes. The concentration of hydroxyapatite crystals is not so high even in an inflammatory joint. The concentrations of putative hydroxyapatite crystals found in the synovial fluid samples varied from about 3 to 18 μg/ml [7]. On the other hand, a high concentration of the crystals is usually needed to elicit a reaction in experimental models: 2 mg/ml of hydroxyapatite crystals was needed to induce inflammation in a rat air-pouch model, an in vivo model [8] and 8 mg/ml of the crystals was necessary to elicit inflammation in an in vitro model [9]. Thus, hydroxyapatite might have more physiological roles than originally thought, one of them being modulation of inflammatory cells. However, the functional significance of hydroxyapatite crystals is, at present, poorly understood.

Because of the important role of the PMNs in responses occurring in microcrystal-induced inflammation [10], we studied the in vitro effects of hydroxyapatite on PMNs. In this report, we show that hydroxyapatite primes PMNs for enhanced chemiluminescence in response to subsequent stimuli.

2. Materials and methods

2.1. Materials

All chemicals were purchased from the Sigma Chemical Co. (St. Louis, Mo, USA) unless indicated. For this study, hydroxyapatite was prepared by Mitsubishi Materials Co., Tokyo, Japan, as described previously [11]. Briefly, it was prepared using a slow precipitation method, and sintered at 1200°C. The crystals were ground down to the desired particle size. The protein content of these particles was checked by the direct Ninhydrin reaction and Bradford's method using Coomassie brilliant blue [12]. No trace of protein appeared. The crystals, all buffers and reconstituted reagents were verified to be pyrogen-free by the limulus amebocyte lysate assay (Sigma Co., St. Louis, MO) following FDA guidelines [13]. All solutions used were determined to contain less than 10 pg/ml of contaminating endotoxin with the exception of the Ficoll-Hypaque separation medium that contained less than 20 pg/ml of contaminating endotoxin. However, this is still less than the reported 100 pg/ml or more of lipopolysaccharide required to prime PMNs for enhanced reactive oxygen metabolites [14]. Confirmation of crystal structure and purity was obtained by high resolution X-ray powder diffraction with monochromatic CuKa radiation on a rotating anode X-ray generator (Rigaku, Model RU-200 and RINT-2400, Tokyo, Japan). Patterns were compared to a known hydroxyapatite standard. The X-ray diffraction patterns of this synthetic hydroxyapatites showed the apatite peaks. Infrared spectra were made of the specimens on an Infrared spectrum's analyzer (Shimadzu, Model FTIR-4000, Kyoto, Japan) operating in the absorbance mode. The specimens exhibited apparently symmetric 630–640 cm^{-1} OH^- stretch peaks.

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Abbreviations: PMNs, polymorphonuclear leukocytes; PBS, Dulbecco's phosphate buffered saline; MEM, Dulbecco's modified Eagle's medium; PMA, phorbol 12-myristate 13-acetate; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine.

SSDI 0014-5793(94)00626-7

2.2. Particle measurements

Crystal sizes were determined using a particle size analyzer (Shimadzu, Model SALD-1100, Kyoto, Japan). The average diameter of the crystal was 3.0 μm. About 3 μm was chosen because of our previous study as described previously [11,15]. The surface areas were measured with a surface area analyzer (Yuasa-Ionics Corp., Model Monosorb. MS-13, Osaka, Japan). One-point Brunauer-Emmett-Teller (BET) surface area determinations were made using nitrogen (adsorbate) in helium (carrier) gas mixtures. The specific surface area for the hydroxyapatite was 6.2 m^2/g. The zeta potential (a measure of the crystal surface charge) was determined using a zeta potential analyzer (Penkem, System-3000) for the hydroxyapatite samples suspended in distilled water. The average zeta potential was −12.2 mV.

2.3. PMNs isolation

PMNs were isolated by Ficoll-Hypaque density centrifugation from heparinized human venous blood, obtained from apparently healthy adults. PMNs were removed separately, washed in Dulbecco's phosphate buffered saline (PBS), and counted. More than 98% of the cells were PMNs, as assessed from Wright–Gimsa stained smears.

2.4. Priming of PMNs with hydroxyapatite

PMNs (5×10^5 cells/ml) were preincubated in Dulbecco's modified Eagle's medium (MEM) at 37°C for varying periods of time with hydroxyapatite particles suspended in PBS or PBS alone. At the end of the pretreatment time, cells were determined to be > 97% viable by the exclusion of Trypan blue. PMN clumping did not occur, as determined by microscopy of cells in suspension. Each stimulus for chemiluminescence was added without removal of the hydroxyapatite particles.

2.5. Chemiluminescence assay

Chemiluminescence was measured in quadruplicate or pentaplicate reaction mixtures, as previously described [11], with an automatic microcomputer-controlled luminescence analyzer (LB 953, Berthold, Germany) at 37°C. The emission curve by each stimulation was recorded continuously for 90 min. Results were expressed as counts per minute (cpm) per 5×10^5 cells, which showed the chemiluminescence intensity integrated from 0 to 90 min. To measure hydroxyapatite-triggered chemiluminescence, PMNs (5×10^5 cells/ml) were incubated with luminol (5-amino, 2,3-dihydro 1,4-phthalazine-dione, 10^{-4} M, Tokyo Kasei Kogyo, Tokyo, Japan) and hydroxyapatite particles. To measure hydroxyapatite-priming for enhanced chemiluminescence in response to triggered by each stimulus, PMNs (5×10^5 cells/ml) were preincubated with hydroxyapatite particles for varying periods of time, and then luminol and each stimulus were added. The following stimuli at pre-determined optimal concentrations were used. (i) *N*-formyl-methionyl-leucyl-phenylalanine (FMLP; 0.1 or 1 μM), (ii) phorbol 12-myristate 13-acetate (PMA; 100 pg/ml), (iii) sodium fluoride (50 μM), (iv) zymosan (1 μg/ml), and (v) the calcium ionophore A23187 (0.1 μM). Cell-free reaction mixtures with hydroxyapatite caused no chemiluminescence responses.

2.6. Statistical analysis

The number (n) of separate experiments from which the data were derived is indicated in the figure legends. Data are expressed as the mean values ± standard error of the mean (S.E.M.). One factor analysis of variance (ANOVA) with repeated measures was used to determine significance between controls and test groups. Differences between treatment means were determined by the Scheffe procedure. The 0.05 level was used as the criterion of statistical significance.

3. Results

Fig. 1 shows the chemiluminescence response, i.e. the production of reactive oxygen metabolites, induced by hydroxyapatite particles in human PMNs. Hydroxyapatite caused a dose-related activation of PMN chemiluminescence that was statistically significant at concentrations of 1,000 μg/ml and greater. Hydroxyapatite at all concentrations tested did not activate chemiluminescence without PMNs.

The priming effects of low-concentrations of hydroxyapatite (less than 10 μg/ml) on FMLP-induced PMN chemiluminescence are shown in Fig. 2. The effects were maximal at 10 μg/ml of hydroxyapatite. Hydroxyapatite priming of PMNs occurred in a concentration-dependent manner. Concentrations of hydroxyapatite that enhance 0.1 μM FMLP-induced chemiluminescence were 2–10 μg/ml. Concentrations of hydroxyapatite that enhance 1 μM FMLP-induced chemiluminescence were 0.2–10 μg/ml: 10 μg/ml hydroxyapatite stimulated chemiluminescence at 0.1 μM or 1 μM of FMLP by 354% or 753%, respectively. The degree of enhancement of FMLP-induced oxidative metabolism was dependent on the length of time the PMNs were incubated with hydroxyapatite (Fig. 3). A maximum (sevenfold) increase in chemiluminescence occurred when PMNs were incubated with hydroxyapatite for 2 h and longer before addition of FMLP (Fig. 3).

The priming effects of low concentrations of hydroxyapatite (0.1–10 μg/ml) on PMA-, sodium fluoride-, zymosan- and calcium ionophore-induced PMN chemiluminescence are shown in Fig. 4. They were maximal at 2 μg/ml with PMA or sodium fluoride, and at 10 μg/ml with zymosan or the calcium ionophore A23187. Preincubation of PMNs with 10 μg/ml hydroxyapatite enhanced the oxidative burst response of these cells to PMA, sodium fluoride, zymosan, or A23187 by 1,354%, 659%, 651% or 316%, respectively.

4. Discussion

We found that hydroxyapatite alone stimulated the PMN respiratory burst but only at high concentrations above 1,000 μg/ml, in agreement with previous reports [9]. We also demonstrated, for the first time, that prein-

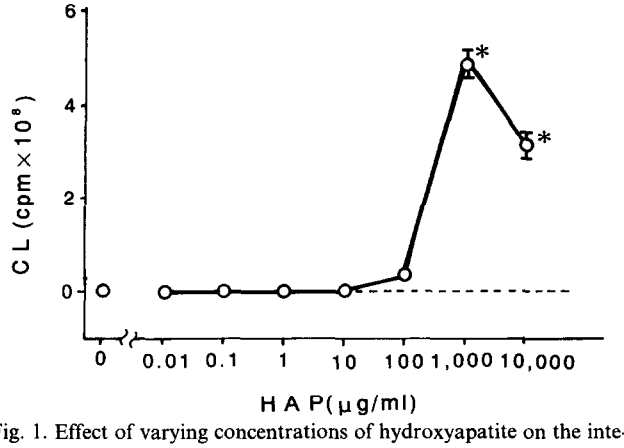

Fig. 1. Effect of varying concentrations of hydroxyapatite on the integral chemiluminescence responses of PMNs. Chemiluminescence is expressed as the mean ± S.E.M., $n = 5$. A statistically significant difference compared with controls is designated by *($P < 0.05$).

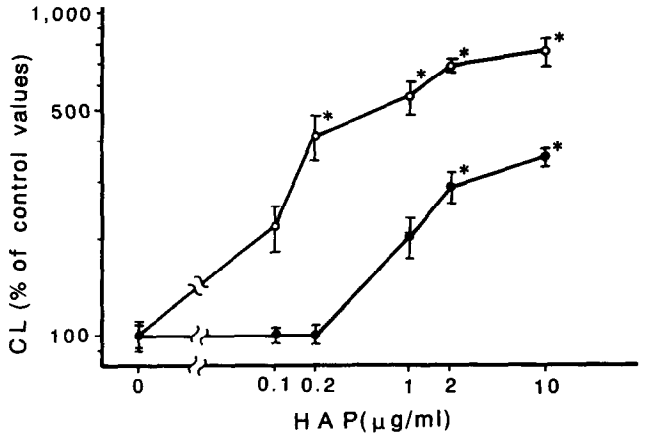

Fig. 2. The priming effect of hydroxyapatite on FMLP-induced PMN chemiluminescence. PMNs were preincubated in the absence or presence of hydroxyapatite at concentrations of 0.1 to 10 μg/ml for 60 min at 37°C and stimulated with 0.1 μM (●) or 1 μM (○) of FMLP. Results are expressed as a % of control values, i.e. chemiluminescence in the absence of hydroxyapatite. Control values were 1,213,333 ± 98,928 and 2,134,667 ± 234,069 for 0.1 and 1 μM of FMLP, respectively (means ± S.E.M., $n = 4$). A statistically significant difference between experiments performed in the absence (control) and in the presence of hydroxyapatite, is designated by *($P < 0.05$).

cubating human PMNs with hydroxyapatite results in enhanced chemiluminescence upon subsequent stimulation by various agents. Levels 300–1, 400% higher than 'stimulus only' controls have been recorded. The priming response, defined as a greater than additive effect of the two agonists alone, is a common feature for most agents which activate PMNs [16]. The priming response is elicited by a variety of agents such as adenosine 5′-triphosphate (ATP) [17], endotoxin [14], stimulatory lipids such as platelet-activating factor (PAF) [18], oleoyl-acetyl-glycerol (OAG) [19], arachidonic metabolites [18], and phorbol esters [20]. However, previous studies on priming have been described only for soluble agents, except for influenza virus [21]. Bautista et al. [22] demonstrated that activation of the liver reticuloendothelial system by in vivo latex phagocytosis enhances the production of oxygen-derived radicals by these cells: they only showed the indirect evidence of the priming effect by an insoluble agent. Our studies here directly examined the potential of an insoluble agent, hydroxyapatite particles, to induce enhanced chemiluminescence. These results demonstrate that hydroxyapatite can be added to the growing list of agents capable of modulating the respiratory burst response of PMNs.

The priming response is associated with a number of events such as degradation, receptor up-regulation, increase in intracellular calcium, and the production of lipid second messengers. Walker and Ward [16] group priming agents into two categories: receptor-dependent and -independent priming. The priming of PMNs by receptor–ligand interactions can be subdivided into two groups. The rapid onset of the primed state shown in Fig. 3 suggests that the priming of PMNs by hydroxyapatite may belong to the first type, in which agonists produce transient increases in intracellular calcium, with responses being mediated by a pertussis toxin-sensitive signal transduction pathway.

Although the significance of these in vitro observations to hydroxyapatite-induced diseases has yet to be fully established, they suggest the following clinical possibility and relationship to the development of hydroxyapatite-induced arthritis and joint loosening after artificial arthroplasty with implants containing hydroxyapatite. PMNs are involved in the inflammatory process in arthritis and joint loosening, and oxygen radicals are known to cause tissue damage. During the spread of hydroxyapatite particles into the lesions, PMNs become primed. Consequently, recruitment of leukocytes to the lesion will include primed PMNs that have a greater inflammatory potential. As the priming effects of low-concentrations of hydroxyapatite on PMN chemiluminescence induced by various stimuli were preserved (Fig. 4), the enhanced generation of oxygen radicals from primed PMNs may play a role in the pathogenesis of tissue damage in hydroxyapatite-induced arthritis and joint loosening after implants containing hydroxyapatite. We have demonstrated two distinct dose-related interactions of hydroxyapatite with human PMNs: low-dose priming and high-dose activation of oxidant generation. Since the concentrations of hydroxyapatite vary in location, it is probable that both priming and stimulating interactions of hydroxyapatite with human

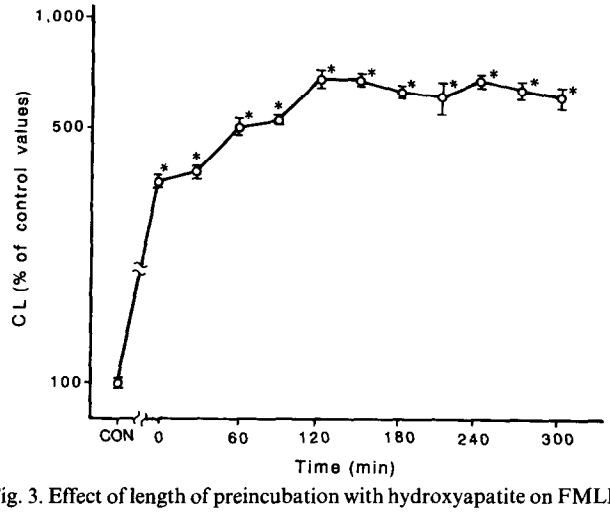

Fig. 3. Effect of length of preincubation with hydroxyapatite on FMLP-induced PMN chemiluminescence. PMNs were preincubated (37°C) for various periods of time with 1 μg/ml of hydroxyapatite or buffer (PBS). Results are expressed as a % of control values, i.e. chemiluminescence in the absence of hydroxyapatite, at each time before addition of 1 μM of FMLP. Control value (CON) without preincubation with hydroxyapatite was 1,128,633 ± 27,954 (means ± S.E.M, $n = 4$). A statistically significant difference between experiments performed in the absence (control) and in the presence of hydroxyapatite at each time, is designated by *($P < 0.05$).

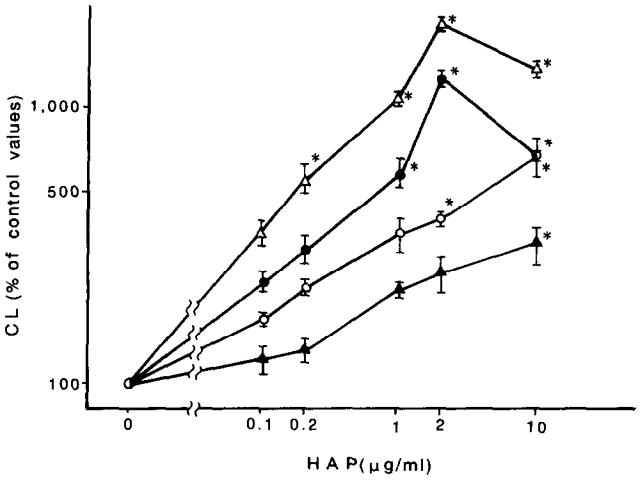

Fig. 4. Priming effect of hydroxyapatite on PMA-, sodium fluoride-, zymosan-, and calcium ionophore-induced PMN chemiluminescence. PMNs were preincubated in the absence or presence of hydroxyapatite at concentrations from 0.1 to 10 μg/ml for 60 min at 37°C and stimulated with 100 pg/ml (△) of PMA, 50 μM (●) of sodium fluoride, 1 μg/ml (○) of zymosan, or 0.1 μM (▲) of the calcium ionophore A23187. Results are expressed as a % of control values, i.e. chemiluminescence in the absence of hydroxyapatite. Control values were 1,124,333 ± 40,683, 2,151,000 ± 84,524, 2,650,000 ± 89,790 and 10,776,667 ± 289,620 for PMA, sodium fluoride, zymosan, and calcium ionophore, respectively (means ± S.E.M., $n = 4$). A statistically significant difference between experiments performed in the absence (control) and in the presence of hydroxyapatite, is designated by *($P < 0.05$).

phagocytes occur in vivo. These interactions of hydroxyapatite with human PMNs may implicate the lower concentration of particles in actual inflammatory sites [7] compared to experimental models [8,9]. We may have been able to see only the direct hydroxyapatite-induced inflammation in previous experimental models.

In conclusion, we first demonstrated that preincubating human PMNs with hydroxyapatite results in an enhanced chemiluminescence upon subsequent stimulation by various agents. We also presented the possibility that the PMNs primed by low-dose hydroxyapatite are important as one of the causes of hydroxyapatite-induced arthritis and joint loosening after artificial arthroplasty with implants containing hydroxyapatite. Additional studies may elucidate the exact role of the hydroxyapatite-primed PMNs in the pathogenesis of the tissue damages.

Acknowledgements: The authors are grateful to Prof. Eiichi Udagawa, Department of Orthopaedic Surgery, Gunma University School of Medicine, for his valuable suggestions; Dr. Hiroyasu Takeuchi and Dr. Toshiyuki Kurosawa, Mitsubishi Materials Co. for kindly providing hydroxyapatite ceramics; Mr. Kiichi Oosawa, Department of Orthopaedic Surgery, Gunma University School of Medicine, and Ms. Kikuko Ishizawa, Department of Internal Medicine, Teikyo University School of Medicine, for their assistance. This work was supported in part by Grant of Japan Orthopaedics and Traumatology Foundation Inc. (JOTF-No. 0057) and Grant-in-Aid for Scientific Research (A) No. 05671199 from the Ministry of Education, Science and Culture in Japan.

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