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Potential Role of the Peroxidase-dependent Metabolism of Serotonin in Lowering the Polymorphonuclear Leukocyte Bactericidal Function

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Serotonin (5-hydroxytryptamine, 5-HT) significantly and dose-dependently suppressed the luminol-enhanced chemiluminescence (CL) signal generated by polymorphonuclear leukocytes (PMN) activated with phorbol myristate acetate (PMA), but did not modify either lucigenin-enhanced CL or the reduction of superoxide dismutase-inhibitable cytochrome c. Moreover, stimulation of PMNs previously incubated with 5-HT resulted in a threefold increase in 5-HT equivalents bound to the proteins of PMN. The addition of catalase or sodium azide substantially reduced this binding. The present results suggest that 5-HT metabolism is mediated by H_2O_2 and myeloperoxidase (MPO) released by activated PMNs. Hence 5-HT could lower the bactericidal function of these cells by competition with hypochlorite formation from halides and MPO/H_2O_2.

Key words: Peroxidase, serotonin, polymorphonuclear leukocytes

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); PMN, polymorphonuclear leukocyte; PMA, phorbol myristate acetate; HRP, horseradish peroxidase; MPO, myeloperoxidase; SOD, superoxide dismutase.

INTRODUCTION

Polymorphonuclear leukocytes (PMN) are the main source of myeloperoxidase (MPO), a heme-containing enzyme which is released into phagocytic vacuoles and the extracellular environment during the degranulation process.[1]

In the presence of hydrogen peroxide (H_2O_2) and halide ions (Cl^-, Br^-), MPO is effective in killing microorganisms.[2] MPO itself has no direct effect on microorganisms but it reacts with hydrogen peroxide (H_2O_2) to form the short-lived compound I, which is highly reactive with oxidisable factors such as halides, thus generating hypohalous acid. These potent oxidants are probably the primary and most important germicidal agents. However, compound I may react with other electron donors and be converted into compound II which does not react with halides.

Endogenous or exogenous compounds, reacting with MPO as electron donors, could compete

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with halides (extracellular chloride concentration of about 103 meq/l) for hypochlorite (ClO^-) production and thus lower the microbicidal activity of PMN. Indeed, MPO-deficient PMNs kill staphylococci three to four times slower than PMNs from normal individuals.[1]

In vitro studies have shown that serotonin (5-HT) and adenine were released by platelets exposed to the cell-free MPO/H_2O_2-halide system[3] or to intact stimulated PMN.[4,5] *In vivo*, the coexistence of PMNs and platelets in the hematovascular system suggests possible interactions between the products of these cells: 5-HT, MPO, O_2^- and H_2O_2.[6]

The ability of human blood to metabolize 5-HT during PMA-stimulated oxidative burst was investigated to determine whether 5-HT metabolism could occur during *in vivo* generation of activated oxygen species (AOS).

We attempted to determine the mechanism of 5-HT metabolism by assessing the ability of 5-HT to interfere with cytochrome c reduction and lucigenin-dependent chemiluminescence,[7] superoxide-dependent reactions, and with luminol-dependent chemiluminescence, a hydrogen peroxide-dependent reaction.[8] Protein binding of 5-HT in isolated PMNs activated by the tumor promotor phorbol myristate acetate (PMA) was also studied.

MATERIAL AND METHODS

Reagents

PMA was purchased from Sigma Chem. Co. (St Louis, USA), stock solutions at a concentration of 1 mg/ml dimethylsulfoxide were stored at −20°C and diluted in PBS before use. HRP was from Boehringer (Mannheim) and H_2O_2 from Merck (Darmstadt), 5-hydroxy [^{14}C] tryptamine creatinine sulphate (5-HT) (50 mCi/mmol) was obtained from Amersham (Les Ulis, France). All other chemicals were generally of the highest available grade and purchased through local chemical suppliers. Distilled water was purified using the Millipore Q system obtained from Millipore (Bedford, Massachussetts).

Isolation of peripheral blood PMNs

Human PMNs were isolated and purified from heparinized (20 U/ml) venous blood (obtained from healthy volunteer donors) by centrifugation of samples over a discontinuous Percoll gradient. A 5 ml volume of 63% Percoll solution in 0.15 M NaCl was layered over 5 ml of 72% Percoll solution in a 15 ml conical tube. Whole blood was layered over the Percoll gradient. The tubes were then spun at 400×g for 20 min at 20°C, resulting in the formation of one band of PMNs above the 72% layer. PMN suspension in Percoll was washed once with an equal volume of physiological serum. Contaminating erythrocytes were lysed by incubation for 10 min in a solution of 130 mM NH_4Cl/10 mM Tris/16 mM K_2CO_3 (pH 7.4) and cells were recovered by centrifugation. The PMNs were washed with PBS (pH 7.4) and then resuspended in the suitable buffer for each experiment. PMN viability was determined by the trypan blue exclusion test and was always greater than 98%.

Chemiluminescence assay

This assay is based on the chemiluminescence (CL) obtained when lucigenin (10,10′-dimethyl bisacridinium-9,9′ dinitrate) or luminol (5-amino-2, 3-dihydrophthalazine-1,4-dione) is reduced by superoxides. CL was monitored in a LKB Wallac model 1251 luminometer, (Wallac Co, Turku, Finland) connected to an Apple computer. PMA (0.1 μg/ml) was used as stimulus. Measurements were carried out every 1 min until maximal CL was obtained.

Assay for release of oxygen-activated species from phagocytes

Human whole blood

A rapid, specific assay developed by Tosi and Hamedani[9] was used to measure superoxide

release from stimulated phagocytic cells in whole blood: 200 µl whole heparinized blood was incubated with different concentrations of 5-HT (10^{-6}–10^{-5} M) and luminol (10^{-4} M) or lucigenin (0.5×10^{-3} M) for 7 min at 37°C in a standard volume of 1 ml PBS, before the addition of PMA (0.1 µg/ml). Controls were performed with cells incubated without 5-HT.

Isolated PMNs

The release of oxygen-activated species from PMNs was studied in the same experimental conditions but using isolated PMNs (10^6 cells) instead of whole blood.

Measurement of PMN superoxide production by cytochrome c reduction

Superoxide dismutase-inhibitable cytochrome c reduction was determined at 37°C using the procedure of Markert *et al.*[10] 5-HT was added to the sample and the reference cuvettes containing the cell suspension (10^6 cells/ml). Upon PMA stimulation (0.1 µg/ml), the change in the rate of cytochrome c reduction was monitored at 550 nm.

Protein binding experiments

Incubation media containing PMNs (10^7 cells/ml), radiolabeled 5-HT (20 µM) and PMA (0.1 µg/ml) were used to determine protein binding. When necessary, superoxide dismutase (SOD, 10 µg/ml), catalase (13000 U/ml) or sodium azide (10 mM) were incubated prior to the addition of PMA. After 20 min incubation at 37°C, proteins were precipitated by the addition of 5% trichloroacetic acid (final concentration) at 0°C. The samples were maintained on ice and then centrifuged. The supernatants were removed and the pellets were continuously washed with 5% trichloroacetic acid and dried using a Skatron combicell harvester prior to determination of protein binding. Covalent binding to protein was determined by liquid scintillation counting with a Beckman liquid scintillation spectrometer.

Measurement of deoxyribose degradation

The reaction mixture (1 ml) contained 2-deoxy-D-ribose 5 mM, 17 µg HRP, 10^{-4} M H_2O_2 and 5-HT ($0–5 \times 10^{-5}$ M) in 0.02 M KH_2PO_4–K_2HPO_4 buffer, pH 7.4. After 30 min incubation at 37°C, 650 U catalase was added to the reaction mixture and incubation was continued for another 10 min. 0.5 ml of 1% (w/v) thiobarbituric acid (dissolved in 0.05 M NaOH) plus 0.5 ml 2.8% (w/v) trichloroacetic acid were added, heated at 100°C for 15 min and cooled. Absorbance of the solution at 532 nm was recorded with an SAFAS high energy spectrometer (SAFAS, Monaco).

RESULTS

5-HT had no effect on lucigenin-enhanced CL from human whole blood, but luminol-enhanced CL was inhibited by incubation of 5-HT with PMA-stimulated human whole blood. Figure 1 shows the luminol-enhanced CL response (panel A) and the lucigenin-enhanced CL response (panel B) to PMA-stimulation of whole blood from a single donor when incubated with 5-HT. Figure 2 summarizes the effects with blood from 6 donors: the luminol-enhanced CL response of PMNs to PMA-stimulation was inhibited in the presence of 5-HT, whereas the lucigenin-enhanced CL response remained unchanged.

As illustrated in Figure 3, similar results were obtained in studies on isolated human PMNs. During the course of the reaction, 5-HT was almost completely oxidized and no longer measurable by HPLC.

Lucigenin-enhanced CL was produced by the reduction of lucigenin by superoxides (O_2^-) released from PMA-stimulated PMNs. To determine, as suggested by the results in Figure 1B and Figure 3, whether 5-HT would actually have no effect on O_2^- production of PMNs, the release of O_2^- by PMA-stimulated PMNs was measured with and without 5-HT using the cytochrome c reduction method. In the 10^{-6} M to 10^{-3} M range, 5-HT

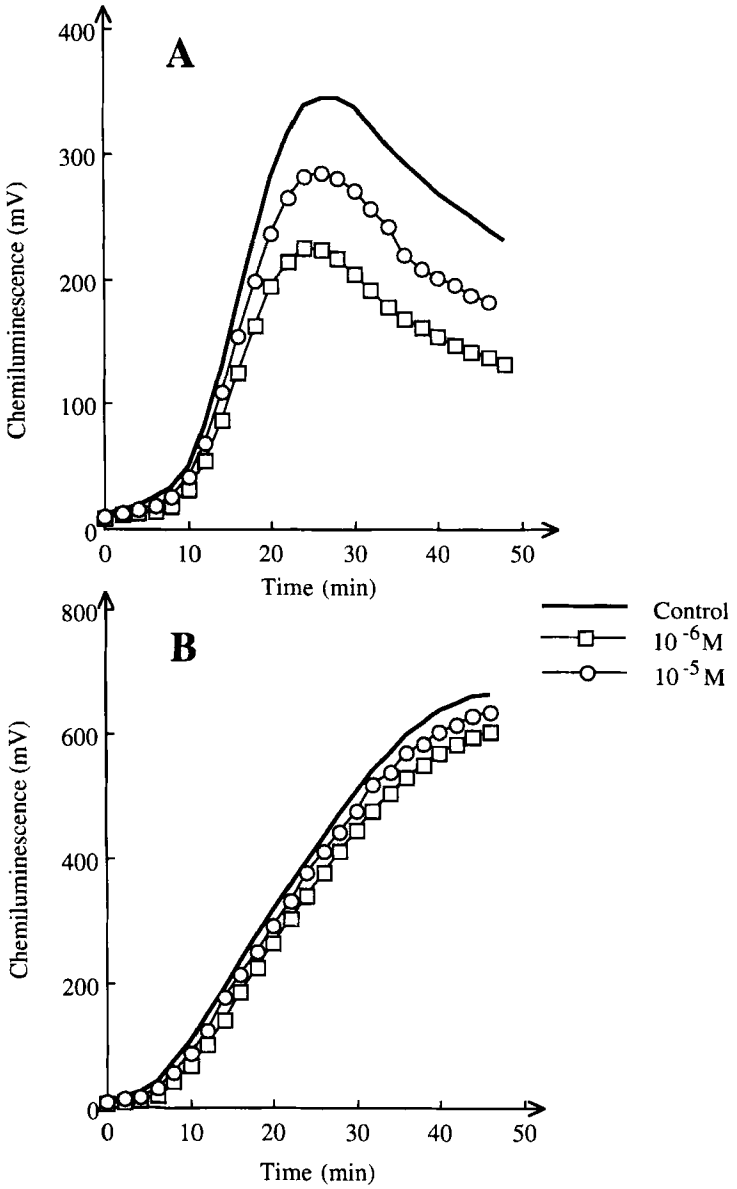

FIGURE 1 Luminol- (A) and lucigenin- (B) chemiluminescence response to 5-HT of human PMA- stimulated whole blood. The figure presents results obtained from whole blood (200 µl) from one donor but is representative of other tested donors (between-donor variation was lower than 12%).

produced no significant inhibitory effect on SOD-inhibitable cytochrome c reduction.

Luminol-enhanced CL was very sensitive to H_2O_2 solution concentrations. To determine the ability of H_2O_2 generated by stimulated PMNs to interfere with 5-HT metabolism, we incubated PMNs, prior to PMA addition, with SOD (which dismutases O_2^- into H_2O_2) and compared the luminol-enhanced CL response of PMNs in the presence and absence of 5-HT (Figure 4).

We also determined the effect superoxide dismutase had on the inhibition of luminol-enhanced

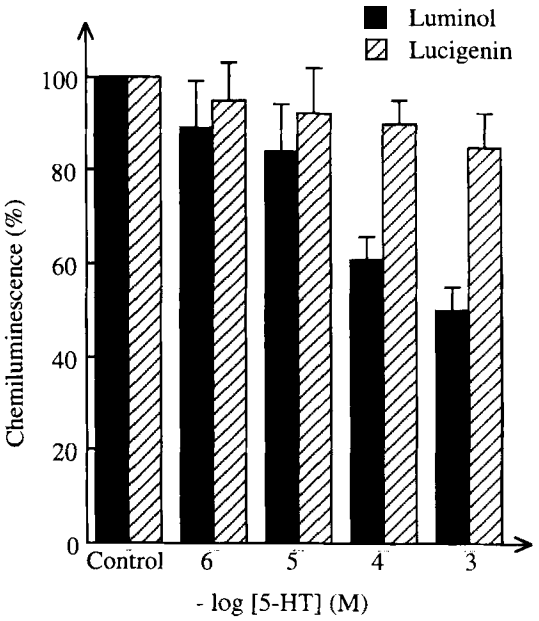

FIGURE 2 Effects of 5-HT on maximum chemiluminescence responses to PMA-stimulated whole blood. Control (100%) corresponds to the response of PMA-stimulated whole blood incubated without 5-HT. The results are expressed as a percentage of control. The means ± SE of 6 separate experiments are shown.

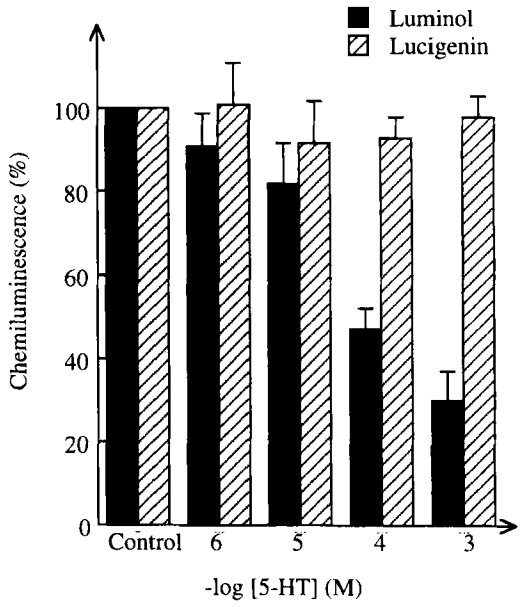

FIGURE 3 Effects of 5-HT on the maximum chemiluminescence response of human PMA-stimulated PMNs (10^6 cells/ml). Control (100%) corresponds to the response of cells incubated without 5-HT (the results are expressed as a percentage of control). The means ± SE of 5 separate experiments are shown.

chemiluminescence by 5-HT. As shown in Figure 4, the addition of SOD to cells, prior to stimulation with PMA, potentiated the effect of 5-HT. Potentiation was maximal with 120 units of SOD. Similar results were obtained in cell-free solutions using HRP and H_2O_2 (not shown). A possible explanation for this result is that superoxide interacts with MPO and limits the ability of 5-HT to inhibit production of hypochlorous acid.[11] Removal of superoxide by SOD would therefore enhance the ability of 5-HT to inhibit production of hypochlorous acid by MPO.

The results of the binding studies of radiolabeled 5-HT to PMN protein during oxidative burst are shown in Figure 5. PMA stimulation resulted in 3-fold increase in protein-bound 5-HT equivalents. The addition of SOD to the incubations appeared to have a slight stimulatory effect, whereas the addition of catalase and sodium azide resulted in a significant (62.5% and 65.2% respectively) reduction in binding. This confirms that the metabolism of 5-HT observed during oxidative burst of human PMNs is peroxidase-mediated. The addition of SOD would stimulate the peroxidase-mediated mechanism through increased H_2O_2 production.

Similar results were obtained in separate studies using HRP and bovine serum albumin (Figure 6). Proteins binding species were formed from [^{14}C] 5-HT by HRP in an H_2O_2-dependent manner and strongly inhibited by both sodium azide and catalase.

The type of protein binding species formed from 5-HT was not determined, but the data presented in Figure 7 suggest that the mechanism probably involves formation of a free radical intermediate.[12] Indeed, deoxyribose is known to be easily disrupted by free radicals, leading to thiobarbituric acid (TBA)-reactive materials which absorb at 532 nm.[13] As shown in Figure 7,

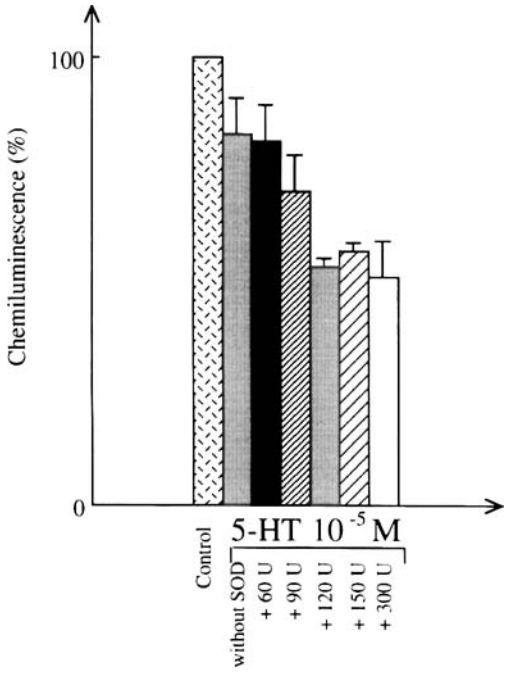

FIGURE 4 Effects of SOD on the maximum luminol-enhanced chemiluminescence response of PMA- stimulated PMNs in the presence of 5-HT (0.2 mM). The means ± SE of 4 separate experiments are shown.

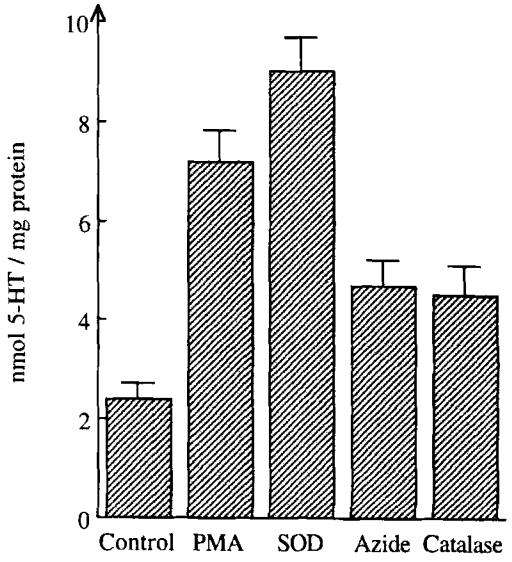

FIGURE 5 Effects of binding of radiolabeled 5-HT to proteins of PMA-stimulated PMNs. PMNs (10^7 cells/ml) were incubated for 20 min at 37°C with radiolabeled 5-HT (20 μM) and PMA (0.1 μg/ml). For comparison, superoxide dismutase (SOD, 10 μg/ml), catalase (13000 U/ml) or sodium azide (10 mM) were incubated with PMNs prior to the addition of PMA. After incubation proteins were precipitated by the addition of 5% trichloroacetic acid (final concentration) at 0°C, centrifuged, washed with 5% trichloroacetic acid and dried. Binding to protein was determined by liquid scintillation counting. Control corresponds to the binding of 5-HT to unstimulated PMNs. The results are expressed as a percentage of the maximum response to PMA stimulation. The means ± SE of 5 separate experiments are shown.

when deoxyribose was incubated with H_2O_2 or HRP or 5-HT or HRP + H_2O_2, 532 nm-absorbance was observed, corresponding to generation of TBA-reactive material. However, in the presence of 5-HT + H_2O_2 + HRP, 532 nm-absorbance was higher corresponding to greater deoxyribose breakdown.

DISCUSSION

PMNs activated by PMA responded immediately by generating AOS which could be measured by luminol- or lucigenin-enhanced CL. In the presence of 5-HT, the luminol-enhanced CL signal was substantially suppressed in a dose-dependent manner.

In the course of the reaction, 5-HT was almost completely oxidized and no longer measurable by HPLC. 5-HT had no effect on SOD-inhibitable cytochrome c reduction, and was bound to PMN proteins. Suppression of the AOS generated by activated PMNs through platelet-derived 5-HT, as shown in luminescence studies, suggested that 5-HT acted as a free radical scavenger. However, sodium azide and catalase inhibition of this effect and of binding to PMN proteins showed that 5-HT metabolism was mediated by H_2O_2 and MPO.

It is possible that 5-HT does not act as a free radical scavenger *in vivo*, but could compete with halides for the MPO/H_2O_2 system of PMNs and lower their bactericidal function. In the light of a very recent report of Panasenko *et al.*[14] showing that ClO^- generated in the MPO/H_2O_2/Cl^- system induced peroxidation of human blood lipoproteins, competition for MPO/H_2O_2 of 5-HT

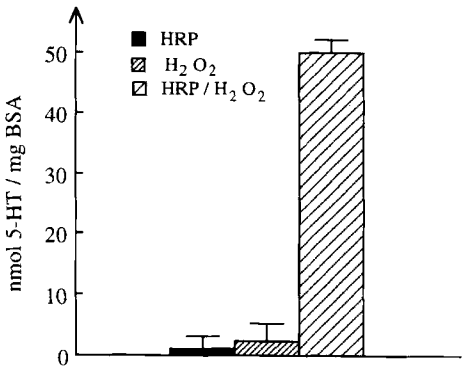

FIGURE 6 Promotion of the binding of radiolabeled 5-HT to BSA by HRP/H_2O_2. A solution of BSA (1 mg/ml) in PBS buffer was added with radiolabeled 5-HT (20 μM), HRP (17 μg/ml) and hydrogen peroxide (1 mM). After 20 min incubation at 37°C, proteins were precipitated by the addition of 5% trichloroacetic acid (final concentration) at 0°C, centrifuged, washed with 5% trichloroacetic acid and dried. Binding to protein was determined by liquid scintillation counting. The means ± SE of 4 separate experiments are shown.

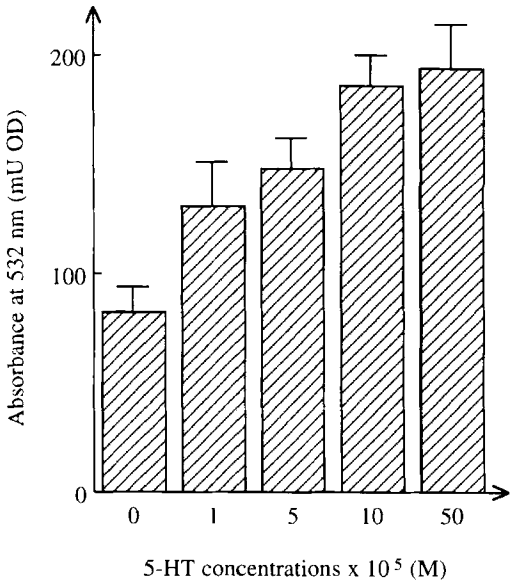

FIGURE 7 Formation of TBA-reactive material by incubating various 5-HT concentrations with HRP/H_2O_2. The reaction mixture (1 ml) contained 2-deoxy-D-ribose 5 mM, 17 μg HRP, 10^{-4} M H_2O_2 and 5-HT (0–5 × 10^{-5} M) in 0.02 M KH_2PO_4–K_2HPO_4 buffer, pH 7.4. After 30 min incubation at 37°C, 650 U catalase was added and incubation was continued for another 10 min. 0.5 ml of 1% (w/v) thiobarbituric acid (dissolved in 0.05 M NaOH) plus 0.5 ml 2.8% (w/v) trichloroacetic acid were added, heated at 100°C for 15 min after cooling, the absorbance of the solution at 532 nm was recorded. The means ± SE of 5 separate experiments are shown.

with Cl^- could also 'protect' lipoproteins. Similar results were obtained when tyrosyl-peptides were exposed to activated PMNs.[15]

Moreover, platelets release 5-HT at inflammation sites and during thrombus formation. The finding that highly reactive binding species are formed from 5-HT during peroxidase-mediated metabolism could thus be essential in understanding cell to cell communications and signaling in the hemovascular systems and related cytotoxic and genotoxic effects in cells.

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