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NITRITE PRODUCTION BY STIMULATED HUMAN POLYMORPHONUCLEAR LEUKOCYTES SUPPLEMENTED WITH AZIDE AND CATALASE

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The formation of nitric oxide by human phagocytes as measured by nitrite production is controversial. We report here that nitrite production by phorbol myristate acetate (PMA)-stimulated human polymorphonuclear leukocytes (PMN) is considerably increased by the addition of azide and a further increase occurs when catalase also is added. Nitrite production by the PMN-PMA-azide-catalase system is unaffected by superoxide dismutase or monomethylarginine but is markedly reduced by the substitution of chronic granulomatous disease for normal neutrophils. The stimulated neutrophils could be replaced by the H_2O_2-generating enzyme system glucose-glucose oxidase. These findings suggest that nitrite production does not, in this instance, reflect nitric oxide synthase activity by human neutrophils but rather the catalase-catalyzed conversion of azide to nitrite in the presence of H_2O_2 generated by the stimulated PMN.

There is considerable interest in the role of nitric oxide and its products in the cytotoxic activity of phagocytes. Reactive nitrogen intermediates (RNI) are formed by murine macrophages as a product of the conversion of L-arginine to L-citrulline by a cytosolic nitric oxide synthase. Formation of nitric oxide by murine polymorphonuclear leukocytes (PMN) also has been reported (1,2). The initial product of synthase activity, nitric oxide, has direct toxic properties; it also can react with oxygen to form nitrogen dioxide (3) and with superoxide to form the peroxynitrite ion (4). The latter can oxidize sulfhydryl groups (5) and, when protonated, decomposes to form a strong oxidant with the properties of hydroxyl radicals (6). Nitrite and nitrate are formed as end products of the metabolism of RNI, with the measurement of nitrite using the Greiss reagent (with or without the reduction of nitrate) being generally employed as a marker of RNI formation.

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Abbreviations: CGD, chronic granulomatous disease; NMA N^G-monomethyl-L-arginine; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocyte; RNI, reactive nitrogen intermediates; SOD, superoxide dismutase.

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The formation of RNI by human phagocytes is controversial. A number of investigators have been unable to detect the formation of nitric oxide by human mononuclear phagocytes *in vitro* (7-12) whereas nitric oxide formation by these cells has been reported in other studies (13). Similarly, nitric oxide formation by human PMN has been detected in some studies (14-17) but not in others (18). We report here on the formation of nitrite by phorbol myristate acetate (PMA)-stimulated human PMN supplemented with azide and catalase. However, the stimulated PMN did not appear to be the immediate source of the nitrite but rather provided the H_2O_2 required for the oxidation of azide by catalase.

MATERIALS AND METHODS

Special reagents. Phorbol myristate acetate and glucose oxidase (Type v-s from *Aspergillus niger*, 950 units/ml without added oxygen) was obtained from Sigma Chemical Co., St. Louis, MO, catalase (CTS 50,500 units/mg) was obtained from Worthington Biochem. Corp., Freehold, NJ, superoxide dismutase (SOD, bovine erythrocytes, 5000 units/mg) was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, and N^G-monomethyl-L-arginine (NMA) was obtained from Schweizerhall Inc. Piscataway, NJ. The catalase and glucose oxidase were heated at 100°C for 20 min. where indicated. The standard salt solution contained 4 x 10^{-3} M sodium phosphate buffer pH 7.4, 0.128 M NaCl, 1.2 x 10^{-2} M KCl, 10^{-3} M $CaCl_2$ and 2 x 10^{-3} M $MgCl_2$.

Isolation of PMN. Human PMN were isolated from normal volunteers and from a patient with chronic granulomatous disease (CGD) as previously described (19). The preparations contained greater than 97% PMN which were greater than 96% viable as measured by trypan blue exclusion.

Nitrite determination. The reaction mixtures (see legend to tables) in a final volume of 0.5 ml were incubated in 12 x 75 mm polystyrene test tubes in a 37°C shaking water bath for 60 min. and nitrite determined by the Greiss reagent as previously described (20).

Statistical analyses. Statistical differences were determined using student's two tailed t test for independent means.

RESULTS

Under the conditions employed in Table 1, a low level of nitrite production was observed on the addition of PMA to human PMN. Nitrite production under these conditions was and when both azide and catalase were added, nitrite production was considerably greater than that observed on the addition of azide alone ($P<0.001$). The stimulatory effect of catalase was lost on its heat inactivation. Nitrite production by PMN + PMA + azide + catalase was abolished by the deletion of the PMN, was greatly decreased but not abolished on the deletion of PMA and was unaffected by the addition of SOD and of NMA at concentrations ranging from 5 x 10^{-5} to 10^{-3} M (10^{-3} M shown in Table 1). Nitrite production was not seen when CGD PMN were combined with PMA and, when azide and catalase were added, nitrite production was considerably lower than that observed with normal PMN ($P<0.001$), but was not abolished. As with normal PMN, SOD had no effect on nitrite production either in the presence or absence of azide and catalase.

When glucose oxidase was added to the CGD PMN-PMA-azide-catalase system, nitrite production increased to the level observed with normal PMN (data not shown). However, under

Table 1. Nitrite production by PMN, PMA, azide and catalase

Supplements	Nitrite (μM)	
None	1.92±0.16(5)	
PMN	1.98±0.12(5)	NS
PMN+PMA	2.33±0.07(5)	<0.05
PMN+PMA+SOD	1.86±0.10(4)	NS
PMN+PMA+catalase	2.12±0.08(3)	NS
PMN+PMA+azide	7.32±0.79(3)	<0.001
PMN+PMA+azide+catalase	22.63±0.52(5)	<0.001
PMN+PMA+azide+heated catalase	7.23±0.49(3)	<0.05
PMA+azide+catalase	1.92±0.09(3)	NS
PMN+azide+catalase	2.87±0.31(3)	<0.05
PMN+PMA+azide+catalase+SOD	22.73±0.39(5)	<0.001
PMN+PMA+azide+catalase+NMA	21.02±5.25(3)	<0.001
CGD PMN+PMA	1.92±0.09(3)	NS
CGD PMN+PMA+SOD	1.92±0.05(3)	NS
CGD PMN+PMA+azide+catalase	3.25±0.08(3)	<0.001
CGD PMN+PMA+azide+catalase+SOD	3.28±0.03(3)	<0.001

The reaction mixture contained the standard salt solution, $2x10^{-3}$ M glucose, and where indicated 5x106 PMN/ml, 100 ng PMA/ml, 10μg SOD/ml, 10μg catalase/ml, 10^{-4} M sodium azide and 10^{-3} M NMA in a final volume of 1.0 ml. The results are the mean ± SEM of the number of experiments shown in parentheses. The P value is for the difference from no supplements (none).

these conditions, PMN and PMA were not required. Table 2 demonstrates the production of nitrite by glucose, glucose oxidase, azide and catalase and the requirement for each component of the reaction mixture. Nitrite production was abolished by heat inactivation of either the catalase

Table 2. Nitrite production by glucose, GO, azide and catalase

Supplements	Nitrite (μM)	
None	-0.47±0.24(4)	
Glucose+GO+azide+catalase	16.19±0.14(4)	<0.001
Glucose deleted	-0.49±0.36(4)	NS
GO deleted	-0.73±0.03(3)	NS
GO heated	-0.66±0.11(2)	NS
Azide deleted	-0.65±0.11(3)	NS
Catalase deleted	-0.62±0.14(3)	NS
Catalase heated	-0.01±0.57(3)	NS
Azide+catalase+H_2O_2	0.48±0.21(4)	<0.02

The reaction mixture contained the standard salt solution and where indicated $2x10^{-3}$ M glucose, 0.2 units glucose oxidase/ml (GO), 10^{-4} M sodium azide, 10 μg catalase/ml and 10^{-3} M H_2O_2 in a final volume of 1.0 ml. The results are the mean ± SEM of the number of experiments shown in parentheses. The P value is for the difference from no supplements (none).

or glucose oxidase and the replacement of glucose and glucose oxidase with reagent H_2O_2 considerably reduced ($P<0.001$) but did not abolish ($P<0.05$) nitrite production.

DISCUSSION

Under our experimental conditions, a small but statistically significant production of nitrite by PMA-stimulated PMN was observed. Total production by 5 x 10^6 PMN during the 60 min. incubation period was 0.41 μM, which was a 21% increase above the background level of 1.92 μM. When azide was added to the PMA-stimulated PMN, nitrite production increased greater than 10 fold to 5.4 μM above background with a further increase to 20.7 μM observed when catalase was also added. SOD, which has been reported to increase the formation of detectable nitrite and nitric oxide by human PMN (16), did not increase nitrite detection by stimulated human PMN in the presence or absence of azide and catalase under our experimental conditions; indeed the small amount of nitrite formed by PMA stimulated PMN was not seen when SOD was added.

These findings indicate that human PMN when stimulated in the presence of azide and catalase produce high levels of nitrite. Does this represent activation of nitric oxide synthase activity in the cell? Our findings suggest that it does not. The initial evidence against such a mechanism was the absence of inhibition of nitrite production by NMA, an arginine analogue which is a competitive inhibitor of nitric oxide synthase activity. Nitrite production by the PMN-PMA-azide-catalase system was markedly reduced when PMN from a patient with CGD were employed, implicating a product of the respiratory burst. The addition of glucose oxidase which, in the presence of glucose, generates H_2O_2 without an apparent superoxide intermediate, reinstituted nitrite production in the presence of CGD PMN, suggesting that H_2O_2 was the required product of the respiratory burst. Indeed, when the H_2O_2-generating enzyme glucose oxidase was added, the stimulated PMN were not required. This suggests that the major if not sole role of the stimulated PMN in the production of nitrite in the presence of azide and catalase is as a source of H_2O_2.

Although the predominant action of catalase is to degrade H_2O_2 to oxygen and water (catalatic activity), catalase also can utilize H_2O_2 to oxidize a number of compounds (peroxidatic activity), particularly when the concentration of H_2O_2 is maintained at a low steady state (21,22). Azide is among the compounds which can be oxidized by catalase in this way (21,23-26). This reaction is favored by an acid pH; at pH 9.0 all the H_2O_2 was decomposed catalatically whereas at pH 5.5, approximately 50% of the H_2O_2 was utilized for the degradation of azide (25). Azide binds to the heme-iron of catalase to form a Fe^{3+}-azide complex which, when exposed to H_2O_2, is degraded with the reduction of the catalase Fe^{3+} to Fe^{2+}. The primary products formed are nitrous oxide and nitrogen (25). However, a small amount of nitric oxide and nitrite is also formed, with the nitric oxide reacting with ferrocatalase to form a complex (21,26). Under our conditions, H_2O_2 generated by glucose and glucose oxidase reacted with catalase to convert azide to a Greiss reagent-reactive species, presumably nitrite. This conversion was considerably reduced when the H_2O_2 generating system was replaced by reagent H_2O_2.

These findings introduce a note of caution in the interpretation of data dealing with the formation of nitrite by phagocytes. It cannot be assumed that the detection of nitrite necessarily

reflects nitric oxide synthase activity in the cells, since other mechanisms for the formation of nitric oxide or nitrite may be operative, particularly when nitrogen containing compounds are added. Stimulation of nitrite production by arginine or inhibition by arginine antagonists would help establish a role for nitric oxide synthase in the nitrite production.

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