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Evidence for generation of a large amount of nitric oxide-like vascular smooth muscle relaxant by cholesterol-rich neutrophils

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To determine the effect of cholesterol incorporation on the ability of neutrophils to generate superoxide radicals and nitric oxide-like vasorelaxant material, isolated human neutrophils were incubated with cholesterol-rich liposomes, which increased total cholesterol content by 141% and esterified cholesterol content by 523%. Cholesterol loading resulted in 5 to 7 fold increase in cytosolic calcium in resting as well as in PMA or f-MLP-stimulated cells, but a marked ($P<0.01$) reduction in both PMA- and f-MLP-stimulated superoxide radical generation by these cells. Nitric oxide-like activity measured as relaxation of rat aortic rings was more pronounced ($P<0.02$) in cholesterol-rich than in cholesterol-poor cells. The greater relaxation of aortic rings in response to cholesterol-rich neutrophils was observed in rings with or without intact endothelium, and was potentiated by superoxide dismutase and inhibited by oxyhemoglobin as well as L-NMMA, thus suggesting that the vasorelaxant material was nitric oxide. The greater generation of nitric oxide by cholesterol-rich neutrophils occurs perhaps in response to increased cytosolic calcium.

Much information has accumulated on the effects of hypercholesterolemia on the biology of platelets, monocytes and vessel walls (1-3); however, there is paucity of data on the function of neutrophils in the presence of hypercholesterolemia. These cells appear in atherosclerotic plaque and in tissues subjected to temporary arterial occlusion, and have the ability to generate free oxygen radicals, proteolytic enzymes and 5-lipoxygenase products, which can injure the endothelium and induce smooth muscle contraction (4,5). Recent studies have shown that human neutrophils generate a vasodilator species, nitric oxide (NO) (6), which also inhibits platelet aggregation (7). NO is destroyed rapidly by superoxide radicals and its activity is decreased in the presence of inhibitors of cyclic GMP (8). This study examines the effect of cholesterol loading on neutrophil NO and superoxide radical generation.

MATERIALS AND METHODS

Cholesterol-free and cholesterol-rich liposomes were prepared as described by Shattil et al (9). Neutrophils were obtained from human peripheral venous blood collected in heparin (10 units/ml) by differential centrifugation (10). The cell population was over 98% pure with over 95% viability. Neutrophils were suspended in HBSS ($1x10^7$ cells/ml). Cholesterol-free or cholesterol-rich liposomes (vol. 0.3 ml) were incubated with 1 ml of cell suspension at 25°C for 1 hr and cell suspensions were then washed to remove the unincorporated liposomes. Studies using ^{3}H-cholesterol showed 25±5% incorporation of radioactivity uptake using this method of loading cholesterol into neutrophils. Total and esterified cholesterol in neutrophils was measured by the method of Zlatski et al (11). The amount of superoxide radicals generated by neutrophils was

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determined by measuring the superoxide dismutase (SOD) inhibitable reduction of ferricytochrome C (12). Intracellular calcium was measured by the method of Minta et al (13) with 5 μM Fluo-3AM as the calcium probe. The effects of cholesterol-rich and cholesterol-poor neutrophils on vascular reactivity were measured in rat aortic rings with (endo+) and and without (endo-) intact endothelium as described previously (10). The aortic rings were precontracted (80% of maximum) with epinephrine and then exposed to 10^5 to 10^7 neutrophils/ml. The nature of the neutrophil-derived smooth muscle relaxant was studied by addition of indomethacin (10μM), the superoxide radical scavenger SOD (200 μg/ml), the cyclic GMP inhibitor oxyhemoglobin (10μM), or the NO synthesis inhibitor L-NMMA (50μM) in the organ bath prior to the suspension of neutrophils. In some experiments, neutrophils were incubated with these agents for 10 min prior to their suspension in the organ bath.

Data relative to the effects of cholesterol loading were evenly distributed and compared by 2-tailed Student's t-test. A P value less than 0.05 was considered significant.

RESULTS

Incubation of neutrophils with cholesterol-rich liposomes resulted in marked ($P<0.01$) increase in total (36.9±8.6 vs 13.9±1.7 μg/10^6 cells) and esterified cholesterol content (26.8±8.9 vs 4.3±1.4 μg/10^6 cells). Superoxide radical generation by cholesterol-rich neutrophils was markedly ($P<0.01$) decreased compared to that by cholesterol-poor cells with the use of either PMA (17.3±7.7 vs 23.9±7.2 nmol/10^6 cells/10 min) or f-MLP (8.4±6.1 vs 11.9±5.5 nmol/10^6 cells/10 min) as the stimulus. Cytosolic calcium in cholesterol-poor cells was 0.3±0.02 μmol/10^5 cells and it increased to 0.9±0.2 and 2.2±1.7 μmol/10^5 cells on stimulation with PMA and f-MLP, respectively. Cytosolic calcium was much ($P<0.01$) higher in cholesterol-rich cells (resting value 1.4±0.1 μmol/10^5 cells, stimulated value 6.5±2.5 and 7.9±2.7 μmol/10^5 cells)

Suspension of cholesterol-poor neutrophils in the organ bath resulted in relaxation of epinephrine-contracted rat aortic segments. Cholesterol-rich neutrophils caused greater ($P<0.02$) relaxation of vascular rings (Fig. 1 and 2, Table I), both in endo+ and in endo- segments. ACh relaxed the endo+, but not the endo- rings. Supernates of cholesterol-free or -rich liposomes did not affect vascular tone in endo+ or endo- rings. The prostaglandin inhibitor indomethacin had no effect on neutrophil-induced vascular relaxation. Superoxide radical scavenger SOD slightly relaxed the endo+ rings, probably due to decreased breakdown of endogenous EDRF (10), and it enhanced ($P<0.02$) the effect of cholesterol-rich neutrophils. The cyclic GMP inhibitor oxyhemoglobin attenuated the neutrophil-induced vasorelaxation and often caused additional contraction (Fig 3). L-NMMA, an inhibitor of NO synthesis, abolished the vasorelaxant effect of neutrophils (Table II). Similar data were obtained whether these inhibitors were present in the organ bath or in the neutrophil suspension.

DISCUSSION

In this study, greater relaxation of rat aortic rings was observed in response to cholesterol-rich than in response to cholesterol-poor neutrophils. The biological characteristics of the vasorelaxant material i.e. its potentiation by SOD, inhibition by oxyhemoglobin and L-NMMA, and no effect of indomethacin, indicate that the vasorelaxant material was NO. Marked neutrophil-induced relaxation in endo- than in endo+ rings confirms results of previous studies (10) and indicates that the relaxant material (NO) was indeed derived from the neutrophils. Greater relaxation of endo- than the endo+ rings suggests that the neutrophil-derived NO may compete with the endothelium-derived NO for activation of guanylate cyclase in vascular smooth muscle .

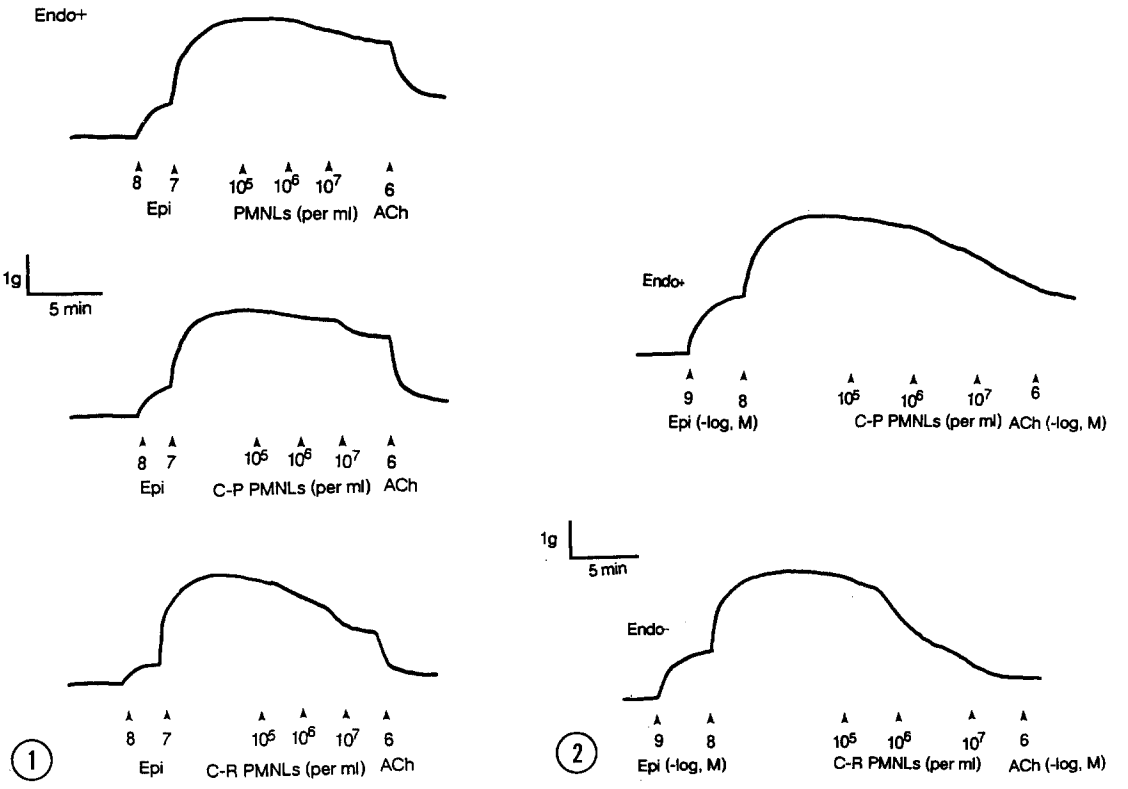

Fig 1. A representative experiment showing effects of untreated polymorphonuclear leukocytes (PMNLs, neutrophils) (top), cholesterol-poor (C-P) PMNLs (middle), and cholesterol-rich (CR) PMNLs (bottom) on vascular rings taken from the same rat thoracic aorta. The endothelium was intact (endo+) in all rings as indicated by relaxation in response to acetylcholine (ACh, 10^{-6}M). Note similar relaxation in response to untreated and C-P PMNLs, but more pronounced relaxation in response to C-R PMNLs.

Fig 2. A representative experiment showing more pronounced vasorelaxant effect of cholesterol-rich (C-R) PMNLs on endo- than on endo+ rings. The intactness of endothelium in endo+ ring is evident from ACh-induced relaxation. Both rings are from the same aortic segment.

TABLE I. *Relaxation of Rat Aortic Rings with Neutrophils*

	Relaxation (%)		
	Concentration of neutrophils (per ml)		
	10^5	10^6	10^7
Endo+ Aortic Rings			
Cholesterol-poor neutrophils (8)	0±5	3±4	13±9
Cholesterol-rich neutrophils (8)	5±7*	11±10*	26±17*
Endo- Aortic Rings			
Cholesterol-poor neutrophils (4)	6±2	28±20	43±28
Cholesterol-rich neutrophils (4)	12±6*	33±31*	60±34*

Data from multiple (# in parenthesis) experiments in mean ±SD.
Relaxation is expressed as decrease (%) in contraction compared to baseline epinephrine-induced force. Cholesterol-rich neutrophils caused greater (*$P<0.02$) relaxation of both endo+ and endo- aortic rings.

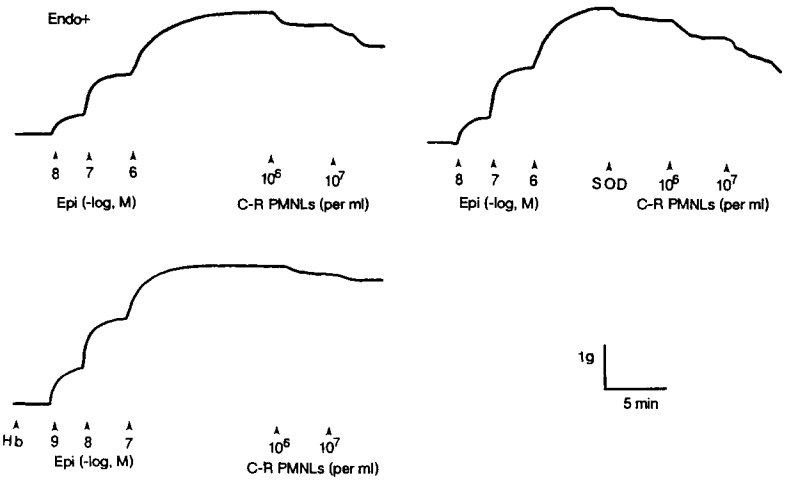

Fig 3. An experiment showing potentiation of the vasorelaxant effect of cholesterol-rich (C-R) PMNLs in the presence of the superoxide radical savenger SOD and inhibition of the vasorelaxant effect in the presence of oxyhomoglobin (Hb).

Increased NO production by cholesterol-rich neutrophils may be due to increased cytosolic calcium, which was seen in resting as well as in PMA- or f-MLP-stimulated cholesterol-rich neutrophils. The increase in neutrophil cytosolic calcium upon cholesterol loading is similar to that seen in platelets, smooth muscle and endothelial cells (14,15). NO generation in endothelial cells increases as cytosolic calcium increases (16,17). Since NO generated by neutrophils is similar to that by the endothelial cells, it is likely that similar calcium-dependent mechanisms are operative in the enhanced synthesis of NO in cholesterol-rich neutrophils.

Intracellular calcium also exists in a dynamic equilibrium with free oxygen species, including superoxide radicals. Release of superoxide radicals is associated with a decrease in calcium flux (19). In other experiments we have observed that an increase in cytosolic calcium in neutrophils (>1 μmol/10^5 cells) induced by increasing calcium content of the buffer causes a decrease in superoxide radical as well as hydrogen peroxide generation. We believe that marked

TABLE II. *Relaxation of (endo+) Rings with Cholesterol-rich Neutrophils : Effect of Various Modulators*

		Relaxation (%) with neutrophils (10^7/ml)	
		Cholesterol-poor	Cholesterol-rich
Control (buffer)	(8)	13±9	26±17
Indomethacin	(4)	15±10	27±5
Superoxide Dismutase	(4)	17±8*	36±9*
Oxyhemoglobin	(5)	-20±14†	-3±5†
L-NMMA	(4)	2±4†	5±5†

Data from multiple experiments in mean ±SD.
Superoxide dismutase enhanced (*P<0.02) the magnitude of neutrophil-mediated relaxation, whereas oxyhemoglobin abolished neutrophil-mediated relaxation and often caused contraction (†P< 0.01).
L-NMMA abolished († P<0.01) the vasorelaxant effect of neutrophils.

increase in cytosolic calcium in cholesterol-rich neutrophils is the basis of decreased superoxide radical generation. In addition release of large amounts of NO released from these cells may also downregulate superoxide radical production (20).

Thus, loading of human neutrophils with cholesterol results in a marked increase in cytosolic calcium, a decrease in superoxide radical generation, and an increase in NO production. As such neutrophils may serve as vasodilatory and platelet inhibitory mediator in hypercholesterolemia. NO generated in large amounts could also be cytotoxic (21) in the process of atherogenesis as well as when the tissues are reperfused.

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