

Formation and release of nitric oxide from human neutrophils and HL-60 cells induced by a chemotactic peptide, platelet activating factor and leukotriene B₄

Harald H.H.W. Schmidt, Roland Seifert and Eycke Böhme

Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69–73, D-1000 Berlin 33, Germany

Received 22 December 1988; revised version received 3 January 1989

Vascular endothelial cells and neutrophils synthesize and release potent vasodilatory factors, i.e. endothelium-derived relaxing factors (EDRF) and neutrophil-derived relaxing factors (NDRF). One EDRF has been identified as nitric oxide (NO) derived from arginine. We studied the synthesis and release of NO from human neutrophils stimulated with the chemotactic peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine, platelet activating factor or leukotriene B₄. The formation and release of NO was enhanced several-fold in the presence of superoxide dismutase, probably by inhibiting superoxide-induced breakdown of NO. The formation and release of NO but not the formation of superoxide anions was decreased in neutrophils pretreated with L-canavanine, an inhibitor of arginine-utilizing enzymes. Our data suggest that at least one NDRF is identical with NO or another labile NO containing compound derived from arginine.

Nitric oxide; Neutrophil-derived relaxing factor; NADPH oxidase; Arginine; (Neutrophil, HL-60 cell)

1. INTRODUCTION

Vascular endothelial cells synthesize and release labile substances among prostacyclin which induce the relaxation of the adjacent vascular smooth muscle [1]. These substances have been termed EDRF, and at least one EDRF stimulates the soluble guanylate cyclase, which catalyzes the formation of cyclic GMP, to a similar extent to nitric oxide (NO) containing compounds [2,3]. One EDRF was recently identified as NO or a labile NO containing compound derived from arginine [4–9].

Correspondence address: H.H.H.W. Schmidt, Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69–73, D-1000 Berlin 33, Germany

Abbreviations: CAN, L-canavanine sulfate; dbcAMP, dibutyryl cyclic AMP; EDRF, endothelium-derived relaxing factors; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; LTB₄, leukotriene B₄; NDRF, neutrophil-derived relaxing factors; 1,25(OH)₂D₃, 1,25-dihydroxy-vitamin D₃; PAF, platelet activating factor; SOD, superoxide dismutase

The basal formation and release of endothelial NO is stimulated by bradykinin and ATP [4,9]. The biosynthesis of NO is inhibited by CAN, a structural analogue of arginine and an inhibitor of various arginine-utilizing enzymes [9].

Rat peritoneal neutrophils induce vascular smooth muscle relaxation via the formation of NDRF which shows pharmacological and physicochemical profiles similar to those of EDRF [10]. In addition, various murine macrophage cell lines synthesize nitrite and nitrate derived from arginine upon exposure to *E. coli* lipopolysaccharide or interferon- γ [11,12]. These oxides of nitrogen have been suggested to play a central role in macrophage-induced cytotoxicity. We investigated the effects of various intercellular signal molecules, which stimulate NADPH oxidase-catalyzed O₂⁻ formation and other functions of neutrophils, on the formation and release of NO. We report here on formation and release of NO from human neutrophils and HL-60 cells differentiated by dbcAMP.

2. MATERIALS AND METHODS

Neutrophils were obtained from heparinized venous blood of male healthy volunteers. The neutrophils were isolated by dextran sedimentation and centrifugation through Ficoll-Hypaque as described previously [13]. Cell preparations consisted of more than 98% viable neutrophils. In some of the experiments, neutrophils were pretreated for 4 h with 2 mM CAN at 18–20°C. HL-60 cells were grown in suspension culture as described recently [13,14]. To induce differentiation along the neutrophil pathway, HL-60 cells were cultured for 48 h in the presence of 0.2 mM dbcAMP [13]. To induce differentiation along the monocytic pathway, HL-60 cells were cultured for 120 h in the presence of 10 nM 1,25(OH)₂D₃ [15]. 1,25(OH)₂D₃ was a kind gift from Drs H. Gutmann and U. Fischer of Hoffmann-La Roche, Basel, Switzerland.

To measure the release of NO, neutrophils (3×10^7 cells) or HL-60 cells (2×10^8 cells) were placed into dialysis tubings (type 20/32-Visking, Serva, Heidelberg, FRG) with an exclusion limit between 8 and 15 kDa. Cells in the dialysis tubings were perfused at a flow rate of 3.3 ml/min with a buffer containing 138 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5.5 mM glucose, 20 mM Hepes/NaOH, pH 7.4, at 37°C, equilibrated with air. The release of NO was determined as described recently [4,7,9]. Briefly, the perfusate was pumped into a 1 l three-necked round bottom flask containing a degassed solution of KI (1%, w/v) in glacial acetic acid under reflux. NO was extracted from this reaction mixture by a constant oxygen-free nitrogen flow (25 l/h) from which a 15 l/h aliquot was drawn through an ice trap into an NO_x-chemiluminescence analyzer (Bendix 8101 C, UPK, Bad Nauheim, FRG). When the background chemiluminescence signal was stable, FMLP, PAF or LTB₄ (each 100 nM) were added to the perfusion buffer, followed by the addition of SOD (50 U/ml). In preliminary experiments, these concentrations of FMLP, PAF and LTB₄ were found to be maximally effective to induce both the formation and release of NO and O₂⁻ (not shown).

NADPH oxidase-catalyzed O₂⁻ release from cells was monitored at 37°C by continuous measurement of ferricytochrome c reduction inhibitable by SOD [13,14]. LTB₄ was a kind gift of Dr Beck of Hoechst AG, Frankfurt/Main, FRG. PAF was purchased from Sigma Chemie (Deisenhofen, FRG). Sources for other materials have been described elsewhere [8,9,13,14].

3. RESULTS

Upon exposure of human neutrophils to the chemotactic peptide FMLP, a NO chemiluminescence signal was detected and reached a plateau after about 30 min, indicating a steady-state formation and release of NO (fig.1). Upon addition of SOD, the NO chemiluminescence signal was potentiated until another steady state was reached after about 20 min. Changing to a buffer without added FMLP and SOD resulted in a rapid decline of the steady-state NO chemiluminescence signal

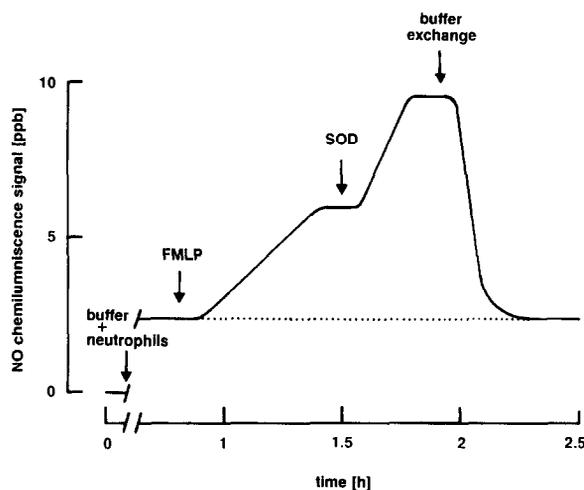


Fig.1. Time course of the detection of the release of NO from human neutrophils. Neutrophils (3×10^7 cells) in a dialysing tube were perfused with a buffer equilibrated with air at 37°C. The release of NO into the perfusate was monitored by the chemiluminescence method described in section 2. FMLP (100 nM) and SOD (50 U/ml) were added to the buffer where indicated by arrows. At the end of the experiment the buffer containing FMLP and SOD was replaced by a buffer devoid of these agents.

to a value identical with that prior to the addition of FMLP and SOD. These data indicate that FMLP induces formation and release of NO which is reversible. Control experiments without added cells revealed that the addition of FMLP or SOD to the buffer in the absence of neutrophils did not affect the NO chemiluminescence signal (not shown).

Similar results to those with FMLP were obtained with PAF and LTB₄ (table 1). In the absence of these stimuli, no NO chemiluminescence signal or O₂⁻ release was detected. FMLP stimulated both the formation and release of NO and O₂⁻. The release of O₂⁻ was about three orders of magnitude higher than that of NO. SOD potentiated the FMLP-induced NO chemiluminescence signal more than 3-fold. When neutrophils were pretreated with 2 mM CAN for 4 h, the FMLP-induced formation and release of NO in the absence and presence of SOD was virtually abolished or reduced by about 40%, respectively. In contrast, FMLP-induced O₂⁻ release was unaffected by CAN. PAF and LTB₄ were much less effective activators of the formation and release of

Table 1

Formation and release of NO and O₂⁻ from human neutrophils and differentiated HL-60 cells

Cell type	NO release (pmol·min ⁻¹ ·10 ⁻⁷ cells)		O ₂ ⁻ release (pmol·min ⁻¹ ·10 ⁻⁷ cells)
	- SOD	+ SOD	- SOD
Neutrophils			
None	<0.5	<0.5	<50
FMLP	20 ± 4	71 ± 27	16800 ± 4700
FMLP + CAN	<0.5**	45 ± 24*	17200 ± 2400
PAF	7 ± 0	35 ± 2	2300 ± 1100
LTB ₄	9 ± 2	24 ± 0	2200 ± 900
HL-60 (dbcAMP)			
None	<0.5	<0.5	<50
FMLP	2 ± 1	5 ± 2	7500 ± 2100
HL-60 (1,25(OH)₂D₃)			
None	<0.5	<0.5	<50
FMLP	<0.5	<0.5	1700 ± 1000

The formation and release of NO and O₂⁻ from human neutrophils and HL-60 cells was determined as described in section 2. HL-60 cells were differentiated along the neutrophil or monocytic pathways with dbcAMP or 1,25(OH)₂D₃, respectively. The formation and release of NO and O₂⁻ was determined under basal conditions and after addition of FMLP, PAF or LTB₄ (100 nM each). In the experiments for the determination of NO formation and release, SOD (50 U/ml) was added to the buffer after the NO chemiluminescence signal had reached a steady state. In some experiments, neutrophils were pretreated with 2 mM CAN for 4 h. Data shown represent the mean ± SE of 3–5 experiments. Data with and without CAN-pretreatment were tested for difference by a paired one-tailed *t*-test. * *p* < 0.1; ** *p* < 0.05

both NO and O₂⁻ than was FMLP. As was the case for FMLP, the PAF- and LTB₄-induced formation and release of NO was potentiated by SOD.

HL-60 cells differentiated along the neutrophil and monocytic pathways with dbcAMP or 1,25(OH)₂D₃, respectively, do not release NO and O₂⁻ in the absence of FMLP. In the presence of FMLP, dbcAMP-differentiated cells released NO and O₂⁻ at rates which were about 10- and 2-fold lower, respectively, than in neutrophils. SOD potentiated the effect of FMLP on the formation and release of NO. Upon exposure to FMLP, HL-60 cells differentiated with 1,25(OH)₂D₃ released O₂⁻ at rates which were more than 4-fold lower than those of dbcAMP-differentiated cells. However, 1,25(OH)₂D₃-differentiated HL-60 cells did not release NO.

4. DISCUSSION

Human neutrophils and dbcAMP-differentiated HL-60 cells form and release NO upon stimulation with FMLP, PAF or LTB₄. The FMLP-induced formation and release of NO from the neutrophils was inhibited by CAN, a structural analogue of arginine and an inhibitor of arginine-utilizing enzymes [9,11]. The latter finding suggests that neutrophil-derived NO is synthesized from arginine as was recently shown for endothelium-derived NO [7,9] and macrophage-derived nitrite and nitrate [11,12]. CAN did not inhibit FMLP-induced O₂⁻ release from neutrophils, indicating that synthesis and/or release of NO and O₂⁻ are independently regulated. Several dissociations between the regulation of the release of O₂⁻ and oxides of nitrogen have been reported for macrophages [11]. The NO chemiluminescence signals from neutrophils and HL-60 cells were increased by SOD, an enzyme scavenging O₂⁻ anions by dismuting them to H₂O₂ and O₂. O₂⁻ anions but not H₂O₂ or H₂O₂-derived radicals are involved in the degradation of EDRF [16]. These data suggest that SOD by scavenging O₂⁻ anions prevents the degradation of neutrophil-derived NO.

Rimele et al. [10] showed the relaxation of isolated rat aortic rings by rat peritoneal neutrophils. These neutrophils were obtained after intraperitoneal injection of oyster glycogen. Thus, it is likely that these cells were stimulated rather than resting. We found that resting human neutrophils obtained from the peripheral blood did not release detectable NO until exposure to appropriate stimuli.

The effectiveness order of intercellular signal molecules to induce the release of NO from human neutrophils is in parallel with the one for O₂⁻ formation and release [17] indicating that the initial steps of the transmembrane signal transduction process are probably identical for either signal molecule. HL-60 cells differentiated with dbcAMP may represent a useful model system to study the mechanisms underlying the formation and release of NO as they show responses to FMLP qualitatively similar to neutrophils. Further studies will have to elucidate the function of human neutrophil-derived NO in vascular smooth muscle regulation and cytotoxicity.

Other recent studies have demonstrated the for-

mation and release of oxides of nitrogen from macrophages [11,12], liver [18], muscular tissue [19] and the formation and release of relaxing factors similar to EDRF from cerebellum [20], respectively. When this manuscript was in the final stage of preparation, Hibbs et al. [21] and Marletta et al. [22] reported that murine macrophages stimulated with *E. coli* lipopolysaccharide also release NO.

We suggest that NO synthesized and released from different cells represents a new inter- and intracellular signal molecule with soluble guanylate cyclase as the intracellular effector system.

Acknowledgements: We thank Professor K.E. Prescher (Institut für Wasser-, Boden- und Lufthygiene, Bundesgesundheitsamt, Berlin, FRG) for providing the facilities to measure NO chemiluminescence and helpful discussion and E. Glaß for help with the cell culture. This work was supported by the Deutsche Forschungsgemeinschaft (DFG); H.H.H.W.S. is the recipient of a DFG postdoctoral fellowship.

REFERENCES

- [1] Furchgott, R.F. and Zawadski, J.V. (1980) *Nature* 288, 373–376.
- [2] Förstermann, U., Mülsch, A., Böhme, E. and Busse, R. (1986) *Circulation Res.* 58, 531–538.
- [3] Ignarro, L.J., Harbison, R.G., Wood, K.S. and Kadowitz, P.J. (1986) *J. Pharmacol. Exp. Therap.* 237, 893–900.
- [4] Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987) *Nature* 327, 524–526.
- [5] Ignarro, L.J., Byrns, R.E., Buga, G.M. and Wood, K.S. (1987) *Circulation Res.* 61, 866–879.
- [6] Furchgott, R.F., Kahn, M.T. and Jothianandan, D. (1987) *Fed. Proc.* 46, 3967.
- [7] Palmer, R.M.J., Ashton, D.S. and Moncada, S. (1988) *Nature* 333, 664–666.
- [8] Schmidt, H.H.H.W., Klein, M.M., Niroomand, F. and Böhme, E. (1988) *Eur. J. Pharmacol.* 148, 293–295.
- [9] Schmidt, H.H.H.W., Nau, H., Wittfoht, W., Gerlach, J., Prescher, K.-E., Klein, M.M., Niroomand, F. and Böhme, E. (1988) *Eur. J. Pharmacol.* 154, 213–216.
- [10] Rimele, T.J., Sturm, R.J., Adams, L.M., Henry, D.E., Heaslip, R.J., Weichman, B.M. and Grimes, D.J. (1988) *J. Pharmacol. Exp. Therap.* 245, 102–111.
- [11] Iyengar, R., Stuehr, D.J. and Marletta, M.A. (1987) *Proc. Natl. Acad. Sci. USA* 84, 6369–6373.
- [12] Hibbs, J.B., Taintor, R.R. and Vavrin, Z. (1987) *Science* 235, 473–476.
- [13] Seifert, R. and Schächtele, C. (1988) *Biochem. Biophys. Res. Commun.* 152, 585–592.
- [14] Seifert, R., Rosenthal, W., Schultz, G., Wieland, T., Gierschik, P. and Jakobs, K.H. (1988) *Eur. J. Biochem.* 175, 51–55.
- [15] Thompson, B.Y., Sivam, G., Britigan, B.E., Rosen, G.M. and Cohen, M.S. (1988) *J. Leukocyte Biol.* 43, 140–147.
- [16] Gryglewski, R.J., Palmer, R.M.J. and Moncada, S. (1986) *Nature* 320, 454–456.
- [17] Dewald, B. and Baggiolini, M. (1986) *Biochim. Biophys. Acta* 888, 42–48.
- [18] Varich, V.Y., Vanin, A.F. and Ovsyannikova, L.M. (1987) *Biofizika SSSR* 32, 1062–1063.
- [19] Martin, W., Smith, J.A., Lewis, M.J. and Henderson, A.H. (1988) *Br. J. Pharmacol.* 93, 579–586.
- [20] Garthwaite, J., Charles, S.L. and Chess-Williams, R. (1988) *Nature* 336, 385–388.
- [21] Hibbs, J.B., Taintor, R.R., Vavrin, Z. and Rachlin, E.M. (1988) *Biochem. Biophys. Res. Commun.* 157, 87–94.
- [22] Marletta, M.A., Yonn, P.S., Iyengar, R., Leaf, C.D. and Wishnok, J.S. (1988) *Biochemistry* 27, 8706–8711.