Nitric oxide-generating system as an autocrine mechanism in human polymorphonuclear leucocytes

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Recent data [López-Farré, Riesco, Moliz, Egido, Casado, Hernando and Caramelo (1991) *Biochem. Biophys. Res. Commun.* **178**, 884–891] revealed that endothelin 1 (ET-1) increases intracellular free $[Ca^{2+}]$ in polymorphonuclear leucocytes (PMN) by a mechanism that can be inhibited by L-arginine. The aim of the present study was to clarify the mechanisms of the interaction between the effects of ET-1 and L-arginine in human PMN. The experimental findings showed that in human PMN: (a) ET-1 and the chemoattractant peptide *N*-formylmethionyl-leucyl-phenylalanine (fMLP) induce both the metabolism of L-arginine to L-citrulline and cyclic GMP (cGMP) formation; (b) the ET-1-induced cGMP production is inhibitable by the L-arginine antagonist *N*^G-monomethyl-L-arginine, there-

fore suggesting the involvement of NO; (c) the ET-1- or fMLP-induced NO/cGMP stimulation is critically dependent on the availability of L-arginine; (d) human PMN possess a L-arginine transport system with both Na⁺-dependent and -independent components; (e) the L-arginine transport system in PMN appears to be feedback-regulated by NO/cGMP in ET-1-stimulated conditions, but not under baseline conditions; (f) the L-arginine transport system in PMN is independent of the γ -glutamyl cycle and is not modified by either ET-1 or fMLP. The L-arginine/NO/cGMP-dependent mechanisms characterized in the present study may be relevant in the regulation of PMN activation in pathophysiological conditions *in vivo*.

INTRODUCTION

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide synthesized by endothelial cells, which is highly expressed in several pathological circumstances, namely myocardial or renal ischaemia and cyclosporin toxicity [1–3]. Several studies have shown that ET-1 represents a constricting signal from the endothelium to contractile cells [4,5]. However, some reports have further suggested that ET-1 may also act on the blood cells [6]. Recently, studies from several laboratories, including ours, have found a significant effect of ET-1 on human polymorphonuclear leucocytes (PMN), either by increasing mobilization of intracellular Ca^{2+} in PMN [7], *N*-formylmethionyl-leucyl-phenylalanine (fMLP)-mediated superoxide anion production [8] and aggregation [9].

PMN are able to produce NO from L-arginine [10–14] and subsequently to increase cyclic GMP (cGMP) production via the soluble guanylate cyclase [15]. cGMP has been regarded as a physiological antagonist of Ca^{2+} -mediated mechanisms in different types of cells [16–18], including macrophages [19,20] and platelets [21]. However, the role of NO/cGMP in monocytes/macrophages [22,23] and PMN [24] is still insufficiently substantiated. In fact, the properties of NO/cGMP on these types of cells include stimulation of chemotaxis [23,24], production of tumour necrosis factor- α [23] and vimentin phosphorylation [25], but also NO/cGMP-mediated blockade of ET-1- or fMLP-induced $[Ca^{2+}]$ transients [7]. Therefore the NO/cGMP-mediated mechanisms in leucocytes are probably of a more complex nature than initially suspected.

We previously reported that L-arginine-mediated NO production acts as a negative-feedback mechanism on the PMN

activation [7]. However, the actual importance of the NO-dependent cGMP generation on PMN activation has not yet been established. In this regard, an interaction between ET-1 and the NO/cGMP-mediated mechanisms is probably of biological relevance.

The aim of the present study was to examine the mechanisms of the interaction between ET-1 and L-arginine/NO/cGMP in human PMN. These interactions were analysed at different levels: (a) the influence of ET-1 on cGMP production; (b) the effect of ET-1 on the metabolism of L-arginine to citrulline; (c) the mechanisms of L-arginine uptake in PMN and the effect of ET-1 on L-arginine uptake, including the feedback regulation of stimulated and unstimulated L-arginine uptake by NO/cGMP-dependent mechanisms. To evaluate further the characteristics and intensity of the response to ET-1, the well-known chemoattractant peptide fMLP was used as an additional PMN activator in several of the experiments.

MATERIALS AND METHODS

Chemicals

Ficoll/Hypaque medium was obtained from Flow Laboratories. Fura-2 acetoxymethyl ester (Fura-2/AM) was from Molecular Probes (Portland, OR, U.S.A.), L-arginine, 8-bromo cGMP (8-Br-cGMP), fMLP, superoxide dismutase (SOD) and L-buthionine sulfoximine (BSO) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium nitroprusside (SNP) was from Merck (Barcelona, Spain). *N*^G-Monomethyl-L-arginine (L-NMMA) was obtained from Calbiochem (La Jolla, CA, U.S.A.), and ET-1 was from Peninsula Laboratories (London, U.K.). cGMP radioimmunoassay kit, [³H]citrulline

Abbreviations used: cGMP, cyclic GMP; 8-Br-cGMP, 8-bromo cGMP; ET-1, endothelin-1; $[Ca^{2+}]$, intracellular free Ca^{2+} concn.; BSO, L-buthionine sulfoximine; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; L-NMMA, *N*^G-monomethyl-L-arginine; PMN, polymorphonuclear leucocytes; PSS, physiological saline solution; SNP, sodium nitroprusside; SOD, superoxide dismutase; MB, Methylene Blue; IBMX, 3-isobutyl-1-methylxanthine.

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and L-[³H]arginine were purchased from Amersham International (Amersham, Bucks., U.K.). All other chemicals were from the highest commercially available quality from Sigma.

PMN preparation

Human PMN were obtained from peripheral blood of healthy donors by Ficoll/Hypaque centrifugation. PMN (95% pure; 98% viable by Trypan Blue exclusion) were resuspended in Hepes buffer, of composition 131 mM NaCl, 4.7 mM KCl, 5 mM glucose and 20 mM Hepes, pH 7.4. PMN were incubated with 0.1 mM L-arginine, 0.1 mM 8-Br-cGMP, 0.1 mM L-NMMA or 0.1 mM L-arginine + 0.5 mM L-NMMA for 60 min at 37 °C.

Measurement of intracellular [Ca²⁺]_i ([Ca²⁺]_i)

PMN (10⁶) were loaded with 2 μM fura-2/AM (60 min at 37 °C) and [Ca²⁺]_i was measured as described previously [7,26,27]. Measurements were done in Ca²⁺-physiological saline solution (PSS), containing 140 mM NaCl, 4.6 mM KCl, 2.0 mM CaCl₂, 1 mM MgCl₂, 10.0 mM glucose and 10 mM Hepes, pH 7.4. Experiments were done in the presence of different agents, to examine the putative mechanisms affecting Ca²⁺ release (for further explanation see the Results section).

Measurement of cGMP

cGMP concentrations were measured as described previously [28] in acetylated samples, by means of an iodinated radio-immunoassay kit (Amersham). In brief, PMN were preincubated in the presence or absence of L-arginine (0.1 mM) for 60 min at 37 °C. After washing twice, PMN were incubated (1 min, 37 °C) in PSS-Ca²⁺ containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 2 mM). IBMX was used in the experiments concerning cGMP to avoid any possible interference by changes in phosphodiesterase activity and to facilitate the accumulation of cGMP. fMLP (0.1 μM), ET-1 (0.1 μM) or SNP (10 μM) was then added and the incubation continued for a further 2 min. In similar samples, L-arginine was co-incubated with the L-arginine antagonist L-NMMA (0.5 mM), or the soluble guanylate cyclase blocker Methylene Blue (MB; 10 μM) [29,30] was added 10 min before ET-1 or fMLP. Additional experiments were carried out in Ca²⁺-free medium supplemented with 2 mM EGTA, to ascertain whether cGMP production was influenced by Ca²⁺ influx.

Uptake of L-[³H]arginine and determination of L-[³H]citrulline content

L-Arginine influx was examined by slight modifications of other methods [31]. PMN (5 × 10⁶ cells/ml) were incubated (30 min, 37 °C) in PSS-Ca²⁺ containing different concentrations of L-arginine and 1 μCi/ml L-[³H]arginine. Additional experiments were carried out by replacing NaCl with equimolar choline chloride in the PSS buffer and in the presence or absence of the L-arginine antagonist L-NMMA (0.5 mM, 60 min preincubation) or the cGMP analogue 8-Br-cGMP (0.1 mM, 10 min preincubation). After sampling for scintillation counting, the supernatants from lysed PMN were evaporated to dryness under N₂ at 37 °C. The extracts were resuspended in 20 mM Hepes/KOH, pH 5.5, and applied to columns of Dowex AG50WX8 (Na⁺

form) which were eluted with water (citrulline fraction) and 0.5 M NaOH (L-arginine fraction) [32]. L-[³H]Arginine and L-[³H]citrulline fractions were quantified by liquid-scintillation counting. In parallel, some samples were spotted on t.l.c. plates (Merck, silica gel). Plates were developed in chloroform/methanol/ammonia/water (1:4:2:1, by vol.) [31]. In these conditions, the *R_f* values for L-[³H]arginine and L-[³H]citrulline were identical to those of standards (0.37 and 0.78 respectively).

Statistical methods

Results are means ± S.E.M. Unless otherwise stated, each value corresponds to a minimum of six experiments done in triplicate. Comparisons were done by analysis of variance and Scheffé's multiple comparisons test. The computer program Enzfitter (Cambridge, U.K.) was used to analyse the kinetics of L-arginine transport. Fitting of the curves was examined by polynomial regression analysis and the Darbin-Watson test (statistical packages Statview and Crisp).

RESULTS

Ca²⁺ mobilization by ET-1 and fMLP

As previously described [7], ET-1 and fMLP provoked a [Ca²⁺]_i transient in PMN ([Ca²⁺]_i: 0.1 μM ET-1 149 ± 15.7 nM; 0.10 μM fMLP 200 ± 12.7 nM; *P* < 0.05 with respect to the baseline). To study the role of mechanisms leading to the formation of endogenous cGMP in the regulation of these [Ca²⁺]_i transients, experiments were done with PMN incubated in medium containing L-arginine (0.1 mM). The ET-1 or fMLP (0.1 μM)-induced [Ca²⁺]_i transients in PMN were markedly decreased in the presence of L-arginine (Table 1). This effect was reversed by co-incubation with the L-arginine antagonist L-NMMA (0.5 mM) (Table 1; [7]) and in the presence of the inhibitor of the soluble guanylate cyclase MB (10 μM), therefore suggesting that the effect of L-arginine is dependent on the activity of the NO synthase and the subsequent cGMP formation. This effect of L-arginine was stereospecific, since no inhibition was observed with 0.1 mM D-Arg (*n* = 3). To confirm more directly the role of cGMP as a regulator of the ET-1- or fMLP-induced [Ca²⁺]_i, PMN were preincubated (10 min) in the presence of 0.1 mM 8-

Table 1 Effect of L-arginine, 8-Br-cGMP, L-NMMA, MB and IBMX on the [Ca²⁺]_i transient induced by ET-1 or fMLP in human PMN

PMN were preincubated in the presence of 0.1 mM L-arginine, 0.5 mM L-NMMA, 10 μM MB or 0.1 mM 8-Br-cGMP before 0.1 μM ET-1 or 0.1 μM fMLP was added. Some experiments were performed in the presence of the phosphodiesterase inhibitor IBMX (2 mM). Results are means ± S.E.M.: **P* < 0.05 with respect to the baseline, †*P* < 0.01 with respect to no agents added, ET-1 or fMLP alone (*n* = 12).

	[Ca ²⁺] _i (nM)		
	Baseline	ET	fMLP
—	144 ± 15	293 ± 15*	344 ± 12*
L-Arginine	136 ± 12	186 ± 12*†	208 ± 8*†
IBMX	136 ± 9	264 ± 12*	305 ± 30*
L-Arginine + IBMX	100 ± 12†	119 ± 14†	148 ± 8*†
L-NMMA	143 ± 15	286 ± 18*	317 ± 26*
L-NMMA + L-arginine	133 ± 12	247 ± 30*	283 ± 38*
MB	137 ± 9	289 ± 17*	309 ± 13*
L-Arginine + MB	132 ± 14	260 ± 40*	301 ± 28*
8-Br-cGMP	102 ± 8†	135 ± 8*†	151 ± 10*†

Table 2 cGMP levels in human PMN activated with 10^{-7} M ET-1

PMN were preincubated in the presence or absence of L-arginine (0.1 mM), L-NMMA (0.5 mM) for 60 min and MB (10 μ M) or SNP (10 μ M) for 10 min before ET-1 was added. All the measurements were done in the presence of IBMX (2 mM). Results are presented as means $\pm$ S.E.M.: * $P < 0.05$ with respect to the baseline; † $P < 0.05$ with respect to L-arginine ($n = 7$).

	cGMP (fmol/ 5×10^6 cells)	
	Baseline	ET
Baseline	113 $\pm$ 6	114 $\pm$ 3
L-Arginine	160 $\pm$ 4*	211 $\pm$ 8*†
L-NMMA	122 $\pm$ 7	119 $\pm$ 6
L-NMMA + L-arginine	105 $\pm$ 5	130 $\pm$ 8
MB	118 $\pm$ 3	122 $\pm$ 9
L-Arginine + MB	109 $\pm$ 9	111 $\pm$ 6
SNP	251 $\pm$ 7*	247 $\pm$ 7*
SNP + L-arginine	239 $\pm$ 6*	253 $\pm$ 9*

Br-cGMP. 8-Br-cGMP significantly blunted the $[Ca^{2+}]_i$ transient induced by ET-1 (0.1 μ M) or fMLP (0.1 μ M) (Table 1).

An inhibitory activity on $[Ca^{2+}]_i$ transients was observed even by using smaller concentrations of L-arginine (inhibition with 10 nM L-arginine $40.1 \pm 7.4\%$; $n = 4$, $P < 0.05$). Since the oxygen free radical, superoxide anion, significantly accelerates NO catabolism, additional experiments were done in the presence of the superoxide anion scavenger SOD, to ascertain further the NO-dependence of the L-arginine-mediated effect. In the presence of SOD (30 units/ml) the inhibition induced by the low L-arginine concentrations was potentiated (inhibition with 10 nM L-arginine + SOD $68.4 \pm 9\%$, $n = 4$; $P < 0.05$ with respect to the inhibition obtained with 10 mM L-arginine alone). The effect of the agents regulating NO and cGMP levels on baseline $[Ca^{2+}]_i$ is also illustrated on Table 1; a small decrease in baseline $[Ca^{2+}]_i$ was observed with L-arginine plus IBMX and with 8-Br-cGMP. As a comparative control and to make the experimental conditions uniform with respect to the studies described in the section below on cGMP, experiments to measure $[Ca^{2+}]_i$ were done in PSS containing 2 mM IBMX alone. No changes in ET-1- or fMLP-induced $[Ca^{2+}]_i$ transients were detected in these conditions compared with the results obtained in the absence of IBMX (Table 1).

Effect of ET-1 or fMLP on cGMP levels

As shown in Table 2, ET-1 (0.1 μ M) did not affect baseline cGMP in PMN. However, cGMP significantly increased when ET-1 or fMLP was added to L-arginine-preincubated PMN (Table 2). Preincubation with L-arginine increased cGMP content in unstimulated PMN, albeit in a smaller proportion (Table 2). In L-arginine-preincubated PMN, the ET-1-mediated cGMP increase was inhibited by co-incubation with the L-arginine antagonist L-NMMA (0.5 mM) and by the inhibitor of soluble guanylate cyclase, MB (10 μ M), therefore suggesting a critical role of the NO synthase/guanylate cyclase sequence. The cGMP increase induced by 10 μ M SNP was not modified by the co-incubation with ET-1 or L-arginine, therefore ruling out any direct influence on the activity of guanylate cyclase itself (Table 2). Similarly to ET-1, fMLP provoked a significant ($P < 0.01$) increase in cGMP in the presence of L-arginine (baseline cGMP 112 ± 2 fmol/ 5×10^6 cells; cGMP in the presence of 0.1 μ M fMLP + 0.1 mM L-arginine 223 ± 7 fmol/ 5×10^6 cells).

Figure 1 Time-dependent L-arginine uptake in human PMN

PMN were incubated in PSS- Ca^{2+} containing L-Arginine (100 μ M) and 1 μ Ci/ml L-[3H]arginine at different times. Results are presented as means $\pm$ S.E.M.: * $P < 0.05$ with respect to zero time ($n = 10$).

Since the activity of the constitutive NO synthase appears to be dependent on calmodulin-bound Ca^{2+} [12,13], and in previous experiments [7] the absence of extracellular Ca^{2+} blunted the ET-1-induced Ca^{2+} peak in PMN, experiments were performed in Ca^{2+} -free medium containing 2 mM EGTA, to study the putative effect of Ca^{2+} on cGMP production. The absence of extracellular Ca^{2+} did not affect the baseline cGMP levels in PMN (cGMP 126.6 ± 16 or 120.3 ± 18 fmol/ 5×10^6 cells in the presence or absence of extracellular Ca^{2+} respectively; $n = 3$ each group; not significant). However, a small but significant decrease was observed in the absence of extracellular Ca^{2+} in the ET-1 + L-arginine-stimulated cGMP increase (cGMP 253 ± 19 or 188 ± 16 fmol/ 5×10^6 cells in the presence or absence of extracellular Ca^{2+} respectively; $n = 4$ each group; $P < 0.05$).

On the basis of the above results, the next series of experiments was carried out to examine whether ET-1 or fMLP would increase NO synthase activity by stimulating the cellular uptake of its substrate, L-arginine.

L-Arginine transport in PMN

First, we characterized the L-arginine transport system in PMN. In the presence of 100 μ M extracellular L-arginine, the L-arginine uptake was linear for approx. 30 min, reaching the steady state between 30 and 40 min (Figure 1). The concentration-dependent L-arginine uptake fitted Michaelis-Menten kinetics, with a V_{max} of 0.11 ± 0.02 μ mol/min per mg of protein and a K_m of 1454 ± 160 μ M (Figure 2a). As shown in Figure 2(b), both a Na^+ -dependent and a Na^+ -independent component of the L-arginine influx were identified. In Na^+ -free medium (Figure 2b), the V_{max} decreased (V_{max} 0.059 ± 0.01 μ mol/min per mg of protein; $P < 0.05$ with respect to Na^+ -containing medium) without changes in the K_m (1635 ± 200 μ M; not significant with respect to Na^+ -containing medium).

Furthermore, the implication of the γ -glutamyl cycle in the transport of L-arginine into PMN was examined. PMN were preincubated (15 min and 4 h) in the presence of 0.1 mM BSO, an irreversible inhibitor of γ -glutamylcysteine synthetase [33]. BSO did not modify the L-arginine uptake by PMN (baseline 0.13 ± 0.03 , BSO 15 min 0.12 ± 0.09 , BSO 4 h 0.14 ± 0.05 μ mol/mg of protein).

Under the above-mentioned conditions, ET-1 (0.1 μ M) did not change either the Na^+ -dependent (results not shown) or the Na^+ -independent L-arginine uptake by PMN (Table 3). This result suggested that the observed increase in cGMP production by ET-1 did not involve an increased L-arginine uptake. However,

Figure 2 Concentration-dependent L-arginine uptake in human PMN

PMN (5×10^6 cells/ml) were incubated (30 min, 37 °C) in PSS- Ca^{2+} containing different concentrations of L-arginine and 1 $\mu\text{Ci}/\text{ml}$ L-[^3H]arginine (a). Additional experiments were carried out by replacing NaCl with equimolar choline chloride in the PSS buffer (b). Insets represent the double-reciprocal plot of the L-arginine transport with (a) and without (b) Na^+ . Double-reciprocal plots are represented as $10^2/\nu$ versus $10^{-2}/[\text{L-Arg}]$. Rates (ν) are expressed as $\mu\text{mol}/\text{min}$ per mg protein. The L-arginine concentrations [S] are in μM . $P < 0.01$ for difference between the curves ($n = 9$).

Table 3 K_m and $V_{\max}$ values of L-arginine transport kinetics in human PMN

PMN were preincubated in the presence of 0.5 mM L-NMMA for 60 min and 0.1 mM 8-Br-cGMP for 10 min before L-[^3H]arginine uptake was performed. The L-arginine transport kinetics were studied at concentrations of unlabelled L-arginine of 100–1000 μM . Results are presented as means $\pm$ S.E.M.: * $P < 0.05$ with respect to L-NMMA alone; † $P < 0.05$ with respect to the baseline ($n = 6$).

	K_m (μM)	$V_{\max}$ ($\mu\text{mol}/\text{min}$ per mg of protein)
Baseline	1454 ± 160	0.11 ± 0.02
ET-1	1532 ± 128	0.14 ± 0.01
L-NMMA	1560 ± 165	0.12 ± 0.02
L-NMMA + ET-1	$158 \pm 25^{\dagger}$	0.19 ± 0.04
L-NMMA + 8-Br-cGMP	1438 ± 45	0.17 ± 0.02
L-NMMA + 8-Br-cGMP + ET-1	1512 ± 54	0.11 ± 0.04

the possibility needed to be raised that a feedback regulation of the L-arginine uptake by the end-product of the pathway, i.e. cGMP, might exist.

To study whether a product-dependent feedback was operative in regulating L-Arg uptake, PMN were preincubated in the presence of 8-Br-cGMP (0.1 mM, 10 min) or the L-arginine antagonist L-NMMA (0.5 mM, 1 h). It should be noted that the presence of L-NMMA in the incubation medium could have a competitive effect with L-arginine for the trans-membrane transport site. Therefore, to ascertain that the putative effect of L-

Table 4 L-[^3H]arginine and L-[^3H]citrulline contents in human PMN stimulated or not with 0.1 μM ET-1 and 0.1 μM fMLP

PMN (5×10^6 cells/ml) were incubated in PSS- Ca^{2+} containing L-arginine (100 μM) and 1 $\mu\text{Ci}/\text{ml}$ L-[^3H]arginine in the presence or in the absence of ET-1 or fMLP. L-[^3H]arginine and L-[^3H]citrulline from lysed PMN were quantified by both t.l.c. and ion-exchange chromatography. The results in this Table correspond to the ion-exchange chromatography. No differences were found between the methods. Results are presented as mean $\pm$ S.E.M.: * $P < 0.05$ with respect to the respective baseline ($n = 6$).

Treatment	Time	L-[^3H]Arginine (nmol/ 5×10^6 cells)	L-[^3H]Citrulline (nmol/ 5×10^6 cells)
Baseline	2 min	45 ± 2	4 ± 2
	30 min	279 ± 9	46 ± 9
ET-1	2 min	45 ± 2	4 ± 2
	30 min	$129 \pm 32^*$	$190 \pm 32^*$
fMLP	2 min	48 ± 3	4 ± 3
	30 min	$149 \pm 15^*$	$172 \pm 48^*$

NMMA was due to its action on the intracellular NO synthase, at the end of the preincubation period extracellular L-NMMA was washed out twice with PSS; in consequence, no L-NMMA was present in the incubation medium at the time of performing the uptake measurements. The L-arginine transport kinetics were not modified in these conditions, showing $V_{\max}$ of 0.12 ± 0.02 $\mu\text{mol}/\text{min}$ per mg of protein and K_m of 1560 ± 165 μM in the presence of L-NMMA, and $V_{\max}$ of 0.10 ± 0.03 $\mu\text{mol}/\text{min}$ per mg and K_m of 1520 ± 193 μM in the presence of 8-Br-cGMP (not significant with respect to baseline). However, when similar experiments were done in the presence of ET-1, significantly lower K_m values were found, therefore indicating that ET-1 actually increased the affinity of the L-arginine uptake system in the presence of NO synthase inhibition (Table 3). No variations in $V_{\max}$ were detected (Table 3). This effect by ET-1, in the presence of L-NMMA, was no longer observed when the PMN were preincubated in the presence of 8-Br-cGMP (10 min, 0.1 mM).

Metabolism of L-[^3H]arginine

To determine whether exogenous L-[^3H]arginine is used in a pathway leading to NO formation in PMN, we studied the accumulation of L-[^3H]citrulline in the presence of ET-1 (0.1 μM) or fMLP (0.1 μM). Generation of L-[^3H]citrulline from L-[^3H]arginine in non-stimulated PMN increased in a small proportion from 2 to 30 min of incubation, as assessed by two different methods, t.l.c. and ion-exchange chromatography (Table 4). Accumulation of L-[^3H]citrulline was significantly increased 30 min after addition of ET-1 or fMLP, with a simultaneous decrease in L-[^3H]arginine levels (Table 4). This effect was reversed by preincubating PMN with 0.5 M L-NMMA (90.5 $\pm$ 6% inhibition), indicating that the conversion of L-[^3H]arginine into L-[^3H]citrulline occurred via the NO synthase pathway (Table 4).

Additional experiments were done to evaluate the role of intracellular L-arginine loading of PMN. As shown in Table 4, when PMN were incubated with L-[^3H]arginine for a short time (2 min) and activated by ET-1 (2 min) or fMLP (2 min), no increase in the L-[^3H]citrulline level was detected. On the contrary, when PMN were preincubated with L-arginine for 30 min, ET-1 (2 min) increased the L-[^3H]citrulline level from 46 ± 9 to 239 ± 18 nmol/ 5×10^6 cells. In consequence, there was a significant difference in the citrulline-production-inducing effect of

ET-1, which did not depend on the duration of ET-1 treatment, but depended on the length of L-arginine pre-loading.

DISCUSSION

The present experiments provide new evidence to clarify the interaction of ET-1 and fMLP with the L-arginine/NO/cGMP-dependent pathways in human PMN. In brief, our results show the following. (a) ET-1 and fMLP induce NO formation in PMN, which leads to an increased production of cGMP by an autocrine effect on the same PMN. (b) The ET-1 or fMLP-induced NO/cGMP stimulation is dependent on the intracellular availability of L-arginine. (c) L-arginine uptake is stimulated by ET-1, although this stimulation by ET-1 becomes evident only in conditions of NO synthase inhibition. The cGMP analogue 8-Br-cGMP reverses the ET-1 effect on L-arginine uptake. (d) The L-arginine transport system in human PMN is independent of the γ -glutamyl cycle, and possesses at least two components, one of them dependent on extracellular Na^+ . (e) NO has an autocrine regulatory function on $[\text{Ca}^{2+}]_i$ levels in PMN, which is dependent on cGMP formation.

PMN produce NO from L-arginine [10–14,24], a process that is enhanced by fMLP [10]. In the present study, we have hypothesized that NO may have an autocrine role in PMN. This hypothesis was supported by the evidence that the $[\text{Ca}^{2+}]_i$ transients were blocked in the presence of L-arginine or 8-Br-cGMP, and by the ET-1- or fMLP-induced elevation of intracellular cGMP levels in L-arginine-incubated PMN. On the contrary, only a small effect of extracellular Ca^{2+} was found on the ET-1-stimulated cGMP generation. In previous experiments we have seen that the ET-1-induced $[\text{Ca}^{2+}]_i$ peak was abolished in the presence of Ca^{2+} -free EGTA buffer. Therefore, this latter result is consonant with reports suggesting that the predominant NO synthase isoenzyme in PMN is independent of calmodulin-bound Ca^{2+} [12,13].

The L-arginine dependence of the cGMP increase and the reversal of the L-arginine effect by L-NMMA both suggested that the effect of ET-1 and fMLP is NO-dependent. Furthermore, the inhibition of this effect by MB demonstrated the implication of the soluble guanylate cyclase. More direct evidence for the presence of this mechanism was obtained in the experiments showing an increased L-citrulline generation in L-arginine-preloaded PMN after stimulation with either ET-1 or fMLP. Although PMN may metabolize L-arginine by arginase [20,34,35], the inhibition of L-citrulline generation by the NO-donor antagonist L-NMMA ruled out the involvement of arginase in the L-arginine conversion into L-citrulline. Additional studies are needed to determine the precise mechanism of NO synthase stimulation by ET-1 and fMLP.

The intracellular concentration of L-arginine appears to be a significant factor for NO synthesis in PMN, as judged by the present series of experiments and as reported by other authors for endothelial cells in L-arginine-deficient conditions [36]. PMN probably became depleted of L-arginine during the isolation procedure, and incubation in the presence of exogenous L-arginine restores the intracellular L-arginine pool. Different authors have reported the importance of local L-arginine levels in macrophage function [19,20], and the present results are further suggestive that changes in L-arginine availability may induce profound changes in PMN biology. From the above results, the existence of a mechanism stimulating L-arginine uptake by ET-1 or fMLP, and coupled to NO formation, was a possibility to be considered. As shown in this study, human PMN transport L-arginine by both Na^+ -dependent and Na^+ -independent transport systems. Cationic or basic amino acids as L-arginine are

transported in several cells by a selective carrier, namely system y^+ , or similar [37]. System y^+ is generally Na^+ -independent, but in some cases, as in renal and intestinal brush-border membranes, it may be partially Na^+ -dependent [37]. Adaptive responses of amino acid uptake have been demonstrated in cells of mesenchymal origin [38]. Transport systems for diverse amino acids have been characterized in blood cells akin to PMN, namely myeloid leukaemia cells and macrophages [39,40]. A different distribution of Na^+ -dependent and -independent systems has been observed, depending on the particular type of amino acids [39,40], but no specific results on L-arginine uptake by PMN have been reported so far. The fact that the interference with the γ -glutamyl system by BSO did not affect the rate of L-arginine uptake supports the presence of a y^+ -type system for L-arginine transport in PMN. A similar transport system has been reported for L-lysine in macrophages [41,42].

The effects of ET-1 and NO/cGMP on the L-arginine transport system in human PMN appeared to interact in a feedback mechanism. The findings that the stimulation of L-arginine uptake by ET-1 is evident only in the presence of the L-arginine antagonist L-NMMA, and that this effect of the L-arginine antagonist is reversed by the cGMP analogue 8-Br-cGMP, both suggest that the putative L-arginine transport system is activated by ET-1 and feedback-inhibited by cGMP. The elucidation of the precise mechanisms of these effects requires further clarification of the characteristics and regulatory sites of the L-arginine transport system.

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