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Characterization of the stable L-arginine-derived relaxing factor released from cytokine-stimulated vascular smooth muscle cells as an N^G-hydroxy-L-arginine-nitric oxide adduct

(inducible nitric oxide synthase/interleukin 1β)

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ABSTRACT **The nature of an L-arginine-derived relaxing factor released from vascular smooth muscle cells cultured on microcarrier beads and stimulated for 20 h with interleukin 1β was investigated. Unlike the unstable relaxation elicited by authentic nitric oxide (NO) in a cascade superfusion bioassay system, the effluate from vascular smooth muscle cells induced a stable relaxation that was susceptible to inhibition by oxyhemoglobin. Three putative endogenous NO carriers mimicked this stable relaxing effect: *S*-nitroso-L-cysteine, low molecular weight dinitrosyl–iron complexes (DNICs), and the adduct of N^G-hydroxy-L-arginine (HOArg) with NO. Inactivation of *S*-nitroso-L-cysteine by Hg^{2+} ions or trapping of DNICs with agarose-bound bovine serum albumin abolished their relaxing effects, whereas that of the vascular smooth muscle cell effluate remained unaffected. In addition, neither *S*-nitrosothiols nor DNICs were detectable in the effluate from these cells, as judged by UV and electron spin resonance (ESR) spectroscopy. The HOArg-NO adduct was instantaneously generated upon reaction of HOArg with authentic NO under bioassay conditions. Its pharmacological profile was indistinguishable from that of the vascular smooth muscle cell effluate, as judged by comparative bioassay with different vascular and nonvascular smooth muscle preparations. Moreover, up to 100 nM HOArg was detected in the effluate from interleukin 1β-stimulated vascular smooth muscle cells, suggesting that sufficient amounts of HOArg are released from these cells to spontaneously generate the HOArg-NO adduct. This intercellular NO carrier probably accounts for the stable L-arginine-derived relaxing factor released from cytokine-stimulated vascular smooth muscle cells and also from other NO-producing cells, such as macrophages and neutrophils.**

Expression of the inducible Ca^{2+}-independent nitric oxide (NO) synthase in cytokine-activated macrophages, neutrophils, and smooth muscle cells leads not only to the formation of NO but also to the simultaneous formation of another considerably more stable L-arginine-derived relaxing factor (1–3). This smooth muscle- or macrophage-derived relaxing factor (MDRF) shares several pharmacological properties with authentic NO; e.g., its relaxing activity is abolished by the NO scavenger oxyhemoglobin or after pretreatment with the soluble guanylyl cyclase inhibitor methylene blue. Moreover, MDRF elicits an increase in the intravascular concentration of cGMP (1, 3). MDRF has on the other hand a significantly longer biological half-life than NO, a molecular mass of <500 Da, and little or no charge at neutral pH (3). From these findings, it would appear that MDRF is an NO-containing substance that activates soluble guanylyl cyclase in the smooth muscle by spontaneously releasing NO.

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Recently, NO carriers, such as low molecular weight dinitrosyl–iron complexes (DNICs) (4) and *S*-nitrosothiols (5), which may be released from NO-producing cells, have been suggested to account for the vasorelaxant properties of endothelium-derived relaxing factor (EDRF) in a cascade superfusion bioassay system but were ruled out due to their greater stability (6). We have investigated whether the MDRF released from cultured rat aortic smooth muscle cells stimulated with interleukin 1β (IL-1β) contains one or several of these NO carriers.

MATERIALS AND METHODS

Materials. Diclofenac (Voltaren) was obtained from Ciba–Geigy; N^G-nitro-L-arginine was from Serva; acetylcholine, bovine serum albumin (BSA)-agarose, L-cysteine, and phenylephrine were from Sigma; oxyhemoglobin was from Calzyme; and human recombinant IL-1β was from Collaborative Research. N^G-Hydroxy-L-arginine (HOArg) was supplied by Glaxo, glyceryl trinitrate was from Pohl-Boskamp, bovine recombinant superoxide dismutase (Peroxinorm) was from Grünenthal, and U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) was from Upjohn.

Authentic NO gas was prepared by the reaction of $FeSO_4$ with sodium nitrite in hydrochloric acid and purified by high-vacuum (10^{-3} mmHg; 1 mmHg = 133 Pa) distillation (7). Low molecular weight DNICs were prepared as described (7). Briefly, evacuated solutions of $FeSO_4$ (3.6 mM) and L-cysteine (72 mM, neutralized with 0.1 M NaOH) were exposed to NO gas (300 mmHg) under agitation. After 5 min, excess NO was removed by evacuation and the green DNIC solution (molar ratio of Fe^{2+} to L-cysteine, 1:20) was immediately frozen in liquid nitrogen. *S*-Nitroso-L-cysteine (Cys-NO) was prepared by exposing L-cysteine (100 mM in double-distilled water) to NO_2 (prepared by mixing NO gas with air) for 10 min at ambient temperature followed by evacuation. The concentration of the Cys-NO solution was estimated by measuring its absorbance at 547 nm (ε = 16.7 $M^{-1} \cdot cm^{-1}$; ref. 8).

Cell Culture. Smooth muscle cells were isolated from the thoracic aorta of male Wistar Kyoto rats [300–350 g (body weight)] by elastase/collagenase digestion and characterized by positive immunostaining with monoclonal antibodies raised against smooth muscle α-actin (9). The cells were serially cultured in Waymouth medium (PAN Systems) containing nonessential amino acids (Biochrom), penicillin (100 units/ml), and streptomycin (100 units/ml), and 7.5% (vol/vol) fetal calf serum (PAN Systems). Upon reaching confluence, cells

Abbreviations: BSA, bovine serum albumin; DNIC, dinitrosyl–iron complex; HOArg, N^G-hydroxy-L-arginine; NO, nitric oxide; MDRF, smooth muscle cell-derived relaxing factor; Cys-NO, *S*-nitroso-L-cysteine; IL-1β, interleukin 1β; EDRF, endothelium-derived relaxing factor.

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were passaged by using 0.05% trypsin/0.02% EGTA. All experiments were performed with cells from passages 8 to 16 seeded into 100-mm i.d. Petri dishes (5×10^6 cells per dish) or onto Cytodex-3 microcarrier beads (Sigma) in T25 flasks (1.5×10^6 cells per 300 μl of beads). Stimulation of NO synthase expression in these cells was achieved by a 20-h incubation in 5 ml of serum-free Waymouth medium containing 0.1% BSA and IL-1β (60 units/ml).

Cascade Superfusion Bioassay. Three endothelium-denuded rabbit aortic rings (≈4 mM wide) were mounted in series between K30 force transducers (Hugo Sachs Elektronik) and a rigid support for measurement of isometric force. The cascade was superfused at a flow rate of 3 ml/min with warmed (37°C) oxygenated (95% O_2/5% CO_2) Krebs–Henseleit solution (pH 7.4; 144.0 mM Na^+/5.9 mM K^+/126.9 mM Cl^-/1.6 mM Ca^{2+}/1.2 mM Mg^{2+}/1.2 mM $H_2PO_4^-$/1.2 mM SO_4^{2-}/25.0 mM HCO_3^-/11.1 mM D-glucose/30 nM superoxide dismutase/1 μM diclofenac/0.1 mM L-arginine) and arranged so that the transit time between rings was 3 s. Passive tension was adjusted over a 30-min equilibration period to 2 g; thereafter, the rings were constricted (6–8 g) with 1 μM phenylephrine. In some experiments, the effluate from a luminally perfused segment of endothelium-intact rabbit aorta (length ≈30 mm, mounted in a thermostatically controlled perfusion chamber) or from a thermostatically controlled disposable chromatography column containing 5×10^6 IL-1β-stimulated smooth muscle cells on beads was used to briefly superfuse the detector tissues. Both the cell column and the aorta were equilibrated separately with Krebs–Henseleit solution by means of a flow distributor, and the path of buffer flow was redirected manually.

Comparative Bioassay. For these experiments, a rabbit or rat aortic ring was mounted at the top of the cascade followed by three other nonvascular and vascular bioassay tissues, such as rat stomach strip (1 g preload), endothelium-denuded rabbit carotid artery (passive tension, 2 g; active tension, 4–6 g) and jugular vein (passive tension, 0.5 g; active tension, 1–1.5 g), and endothelium-denuded porcine coronary artery (passive tension, 3–5 g; active tension, 8–10 g). For the nonvascular smooth muscle preparations, changes in length rather than isometric force were recorded by using TF3V3 lever transducers (Hugo Sachs Elektronik). The bioassay tissues were preconstricted with a combination of phenylephrine (1 μM) and acetylcholine (1 μM, for the rat stomach strip) or U46619 (0.1 μM, for the porcine coronary artery).

HPLC Analyses. HPLC analysis of the HOArg-NO adduct was performed with a 250 × 4 mm (i.d.) LiChrospher 100 NH_2 column (Merck), and material was isocratically eluted at ambient temperature with 10 mM KH_2PO_4, pH 4.3/acetonitrile/methanol, 27.5:70:2.5 (vol/vol). The flow rate was set to 1.5 ml and the UV absorbance of the eluate was continuously monitored at 205 nm. L-Citrulline, L-arginine, and HOArg were eluted at 7.6, 15.0, and 17.1 min, respectively.

HPLC/fluorescence analysis of the concentration of L-citrulline, L-arginine, and HOArg in the supernatant or lysate of IL-1β-stimulated smooth muscle cells was performed by precolumn derivatization with *o*-phthalaldehyde as described (10), except that material on the column [250 × 4.6 mm (i.d.) UltraTechsphere ODS, HPLC Technology] was isocratically eluted with 10 mM KH_2PO_4, pH 5.85/acetonitrile/methanol/tetrahydrofuran, 79:10:10:1 (vol/vol) at a flow rate of 1 ml/min.

ESR Spectroscopy. ESR spectra were recorded at 77 K with a Bruker (Billerica, CA) model EPR 300E (instrument settings: frequency, 9.33 GHz; high-field modulation, 100 kHz and 0.5 mT; microwave power, 20 mW; time constant, 0.05 s). Samples (0.6 ml, aqueous solution) were placed in a quartz Dewar vessel (5 mm i.d.), which was chilled with liquid nitrogen (4). DNIC concentrations were calculated by comparison with the ESR signal of a DNIC-cysteine standard based on double integration of the first-derivative of the ESR signals (4).

Determination of NO_2^- and *S*-Nitrosothiols. NO_2^- determinations were performed as described (11). The concentration of *S*-nitrosothiols was estimated from the difference in absorbance (546 nm) of the samples in the presence and absence of 0.3 mM $HgCl_2$.

Statistics. Unless indicated otherwise, all data in the figures and text are expressed as the mean ± SEM of *n* experiments with different batches of smooth muscle cells. Statistical evaluation was performed by one-way analysis of variance followed by a Bonferroni post test for multiple comparisons with a *P* value of <0.05 considered statistically significant.

RESULTS

Characterization of the Relaxant Activity of MDRF. Superfusion of the detector tissues with the effluate from an endothelium-intact acetylcholine (1 μM)-perfused segment of rabbit aorta, likewise authentic NO (Fig. 1), elicited only a relaxation of the first detector tissue in the cascade. In contrast, short-term (≤20 s) superfusion with the effluate from a column of IL-1β-stimulated smooth muscle cells on beads, but not from unstimulated cells (data not shown), resulted in a relaxation of all three detector tissues that were separated from the column by a transit time of 1, 4, and 7 s, respectively (Fig. 1). The same MDRF-type relaxant response was obtained when 0.5 ml of the effluate was collected into a vial and an aliquot (50 μl) thereof was applied to the bioassay cascade (Fig. 2). Addition of oxyhemoglobin (10 nM) to the superfusate abolished the relaxing effect of MDRF ($n = 3$). Its biological half-life when incubated in Krebs–Henseleit solution for various times was estimated to be 19 ± 4 min at 0–4°C and 12 ± 2 min at 20°C ($n = 3$), as judged by the relaxation of the third detector tissue that did not respond to authentic NO (Fig. 1 *Inset*). Lyophilization of the effluate from the column of IL-1β-stimulated smooth muscle cells on beads resulted in a complete loss of its relaxing activity.

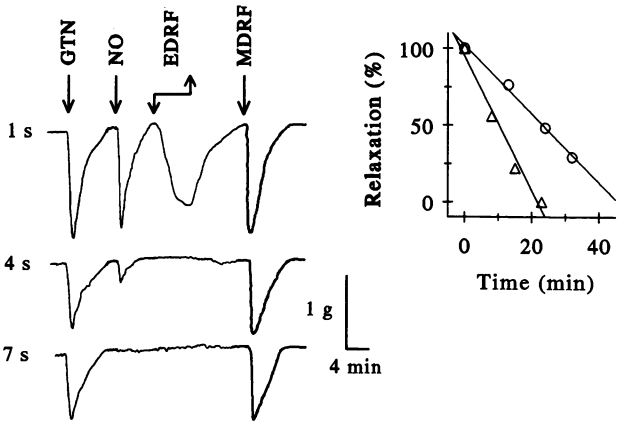

FIG. 1. (*Left*) Comparison of the vasorelaxant properties of glyceryl trinitrate (GTN, 100 pmol), NO (500 pmol), EDRF (generated by an endothelium-intact luminally perfused segment of the rabbit aorta in response to 1 μM acetylcholine), and the effluate (15-s superfusion) from a column of IL-1β-stimulated smooth muscle cells on beads (MDRF). Typical traces representative of five further experiments are shown. (*Right*) Estimation of the biological half-life of MDRF. Aliquots of the effluate from the column of IL-1β-stimulated smooth muscle cells (100 μl) were collected, incubated in Krebs–Henseleit solution at 0–4°C (○) or 20°C (△) for the indicated periods of time, and then applied to the superfusion bioassay cascade. The resulting relaxation of the third detector tissue, which did not respond to authentic NO, is expressed as percentage of the relaxant response elicited by the same volume of the effluate administered immediately after its collection (0 min, taken as 100% value).

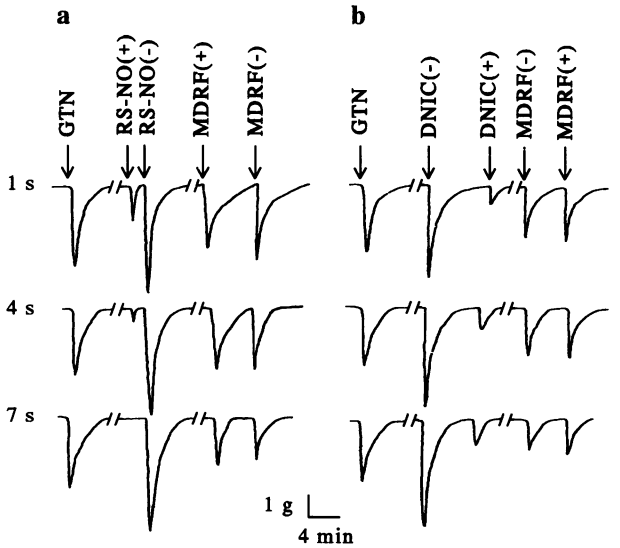

FIG. 2. (*a*) Effects of pretreatment with $HgCl_2$ on the vasorelaxant properties of Cys-NO (125 pmol, RS-NO) and MDRF (50-μl effluate from IL-1β-stimulated vascular smooth cells). The effluate (0.5 ml) or 1.25 nmol of Cys-NO in 0.5 ml of Krebs–Henseleit solution was mixed with 5 μl of $HgCl_2$ (118 mM) for 1 min at ambient temperature, and 50 μl of either solution was applied to the bioassay cascade. +, $HgCl_2$ pretreatment; −, no pretreatment. (*b*) Effects of agarose (−) or BSA-agarose (+) pretreatment on the relaxant response to DNICs (150 pmol) and MDRF (50-μl effluate from IL-1β-stimulated vascular smooth muscle cells). The effluate (0.5 ml) or 1.5 nmol of DNICs in 0.5 ml of Krebs–Henseleit solution was mixed with 200 μl of agarose or BSA-agarose (≈120 μg of immobilized BSA) for 2 min at ambient temperature. Thereafter, the mixtures were briefly centrifuged and 70 μl of the supernatant was applied to the bioassay cascade. Typical traces representative of four further experiments are shown. GTN, 30 pmol of glyceryl trinitrate.

DNICs and *S*-Nitrosothiols Do Not Account for the Biological Activity of MDRF. To elucidate the chemical nature of MDRF, we compared its relaxing activity with that of three putative NO carriers: DNIC, Cys-NO, and the adduct HOArg-NO (12). At submaximal doses, all three compounds indeed caused a relaxant response that was similar to that elicited by MDRF (Figs. 2 and 3).

S-Nitrosothiols are rapidly inactivated by Hg^{2+} ions, resulting in the formation of NO_2^- (11). Addition of $HgCl_2$ to the effluate from the column of IL-1β-stimulated smooth muscle cells on beads had no effect on its relaxing activity, while that of Cys-NO was strongly reduced (Fig. 2*a*). $HgCl_2$ itself had no effect on vascular tone, and the same results were obtained when *S*-nitrosoglutathione (250 pmol) was used instead of Cys-NO ($n = 3$). Moreover, no difference in the concentration of NO_2^- in the effluate was detected (≈0.1 μM) in the presence or absence of $HgCl_2$, even after concentrating the effluate by lyophilization (≈1 μM NO_2^-; $n = 6$).

Labile low molecular weight DNICs can be trapped by BSA to yield stable high molecular weight DNICs (4). Incubation of MDRF with immobilized BSA had no effect on its relaxing activity, while that of the DNIC solution was markedly attenuated (Fig. 2*b*). BSA-free agarose beads, on the other hand, had no effect on the biological activity of the DNIC solution. Moreover, DNIC was not detected in the effluate from the column of IL-1β-stimulated smooth muscle cells on beads by ESR spectroscopy, even after lyophilization ($n = 6$), suggesting that if DNIC had been released at all, its concentration in the effluate was well below the detection limit (0.1 μM; i.e., ≤0.01 μM).

Characterization of MDRF as the HOArg-NO Adduct. Equimolar amounts of HOArg and sodium nitrite (1 μmol) were incubated in bidistilled water (0.1 ml, pH 2.5 due to the use of the dihydrochloride salt of HOArg) for 10 min at 0–4°C.

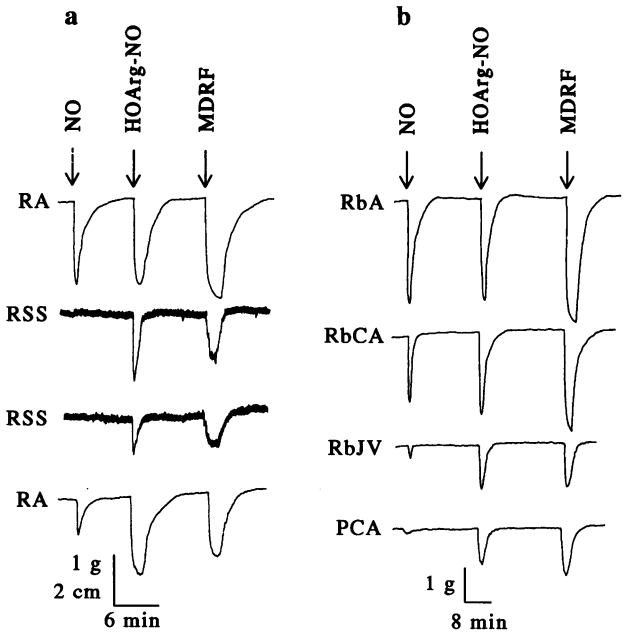

FIG. 3. Comparative bioassay of the vasorelaxant properties of authentic NO (2 nmol), the HOArg-NO adduct (0.3 nmol), and MDRF (20-s superfusion with the effluate from IL-1β-stimulated vascular smooth muscle cells) with different vascular and nonvascular smooth muscle preparations. Typical original traces representative of three further experiments are shown. RA, rat aorta; RbA, rabbit aorta; RbCA, rabbit carotid artery; RbJV, rabbit jugular vein; RSS, rat stomach strip; PCA, porcine coronary artery.

Separation of the reaction products (200 nmol equivalent to 20 μl of the mixture) by normal-phase HPLC revealed the quantitative formation of a new peak that was well separated from HOArg at 18.7 min. The corresponding HPLC fraction showed an UV maximum at 320 nm, typical for an adduct of NO with the guanidino moiety (12, 13), and the same relaxing activity as the mixture of HOArg and acidified NO_2^- that was stable down the bioassay cascade. Appropriate dilution of the mixture with Krebs–Henseleit solution to match the vasorelaxant effect of NO or MDRF revealed that the HOArg-NO adduct was ≈10 times more potent than NO (Fig. 3), presumably because of its greater stability. In contrast, much higher doses of either HOArg or NO_2^- (50 nmol compared to 25–300 pmol of the adduct) had no effect on vascular tone ($n = 6$). Moreover, superfusion of the detector tissues with 10 μM HOArg led to a marked stabilization of the relaxant response to NO ($n = 4$).

To compare the pharmacological profile of MDRF and the HOArg-NO adduct, different vascular and nonvascular smooth muscle preparations that responded to either authentic NO or the HOArg-NO adduct were employed in addition to the rabbit aorta. As shown in Fig. 3*a*, submaximal doses of MDRF and the HOArg-NO adduct elicited equivalent relaxant responses of the rat aorta and the rat stomach strip that, on the other hand, was largely insensitive to authentic NO. Both MDRF and the HOArg-NO adduct also relaxed the rabbit carotid artery, the rabbit jugular vein, and the porcine coronary artery to a similar extent (Fig. 3*b*).

Formation of HOArg by IL-1β-Stimulated Smooth Muscle Cells. The possible formation of HOArg by IL-1β-stimulated smooth muscle cells was investigated by reverse-phase HPLC analysis. After a 20-h exposure to IL-1β, there was a significant increase in the concentration of NO_2^- in the conditioned medium (Fig. 4) that was abolished in the presence of the NO synthase inhibitor, N^G-nitro-L-arginine (600 μM, equivalent to the initial concentration of L-arginine in the medium). This increased NO_2^- production was paralleled by an increase in the

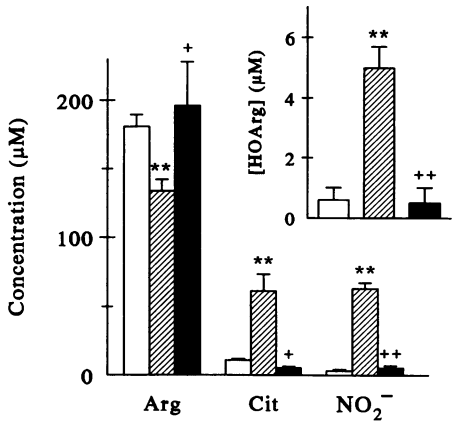

FIG. 4. Concentration of L-arginine (Arg), L-citrulline (Cit), and NO_2^- in the supernatant of cultured smooth muscle cells after a 20-h incubation in the absence (open bars, $n = 5$) or presence (hatched bars, $n = 5$) of IL-1β at 60 units/ml. The solid bars represent the values obtained with IL-1β-treated smooth muscle cells incubated in medium containing 600 μM N^G-nitro-L-arginine ($n = 3$). (*Inset*) Corresponding changes in the concentration of HOArg in the supernatant. **, $P < 0.01$ vs. control; +, $P < 0.05$ vs. IL-1β; ++, $P < 0.01$ vs. IL-1β.

concentration of L-citrulline, the magnitude of which matched the decrease in the level of L-arginine. Moreover, there was a 10-fold increase in the level of HOArg in the conditioned medium of IL-1β-treated cells to ≥5 μM, but not in that of control cells or IL-1β-activated cells treated with the NO synthase inhibitor (Fig. 4 *Inset*). However, HOArg was not detectable in the lysate of IL-1β-stimulated smooth muscle cells, although the intracellular concentration of L-citrulline (estimated on the basis of an average cell volume of 1 pl) rose from 120 to 810 μM and L-arginine levels remained virtually constant at 120 μM.

In contrast to control cells, IL-1β-treated smooth muscle cells grown on beads and incubated in L-arginine (1 mM)-supplemented Krebs–Henseleit solution for 30 min at 37°C also produced HOArg. The concentration of HOArg in the supernatant increased from <0.1 to ≥1 μM (data not shown).

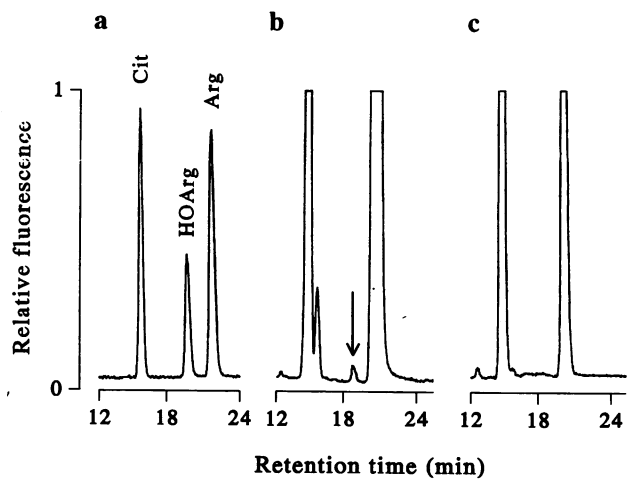

FIG. 5. (*a*) Separation of the *o*-phthalaldehyde derivatives (each at 100 pmol) of authentic L-citrulline (Cit), L-arginine (Arg), and HOArg by HPLC/fluorescence detection. (*b* and *c*) HPLC analysis of the effluate (100 μl) from the same batch of smooth muscle cells on beads cultured in the presence (*b*) or absence (*c*) of IL-1β (60 units/ml) for 20 h and superfused with Krebs–Henseleit solution containing 0.1 mM L-arginine. The arrow in *b* indicates the position of HOArg in the chromatogram, the peak area of which corresponds to ≈7 pmol. Similar findings were obtained with five other batches of smooth muscle cells.

Moreover, significant concentrations of HOArg (up to 100 nM) were detected in the effluate from these cells (Fig. 5).

Decomposition of the HOArg-NO Adduct Upon Derivatization with *o*-Phthalaldehyde. HPLC analysis of a freshly made solution of the HOArg-NO adduct (300 pmol) revealed that at least 52.6% of the adduct decomposed during the derivatization procedure. HOArg itself (91.8%) represented the bulk of the decomposition products followed by L-citrulline (5.0%) and L-arginine (3.2%). The remaining 47.4% of the HOArg-NO adduct either was not detectable due to lack of derivatization or decomposed into substances that cannot be eluted from the HPLC column or react with the *o*-phthalaldehyde reagent. In this context it is important to note that apart from *o*-phthalaldehyde and other additives, the reagent (pH 10.4) also contains 2-mercaptoethanol, a strong reducing agent.

DISCUSSION

The present findings demonstrate that IL-1β-stimulated vascular smooth muscle cells generate an L-arginine-derived relaxing factor (MDRF) that appears to be distinct from NO or EDRF due to its greater stability in a cascade superfusion bioassay. The relaxant effect of MDRF was abolished in the presence of the NO scavenger oxyhemoglobin, suggesting that it is mediated by the spontaneous release of NO from this compound. Both low molecular weight DNICs and *S*-nitrosothiols, such *S*-nitroso-L-cysteine and *S*-nitrosoglutathione, mimicked the stable relaxant response to MDRF. These NO carriers have been proposed to account for the vasorelaxant properties of EDRF (4, 5); however, their pharmacodynamic profiles clearly differ from that of authentic NO or EDRF in the cascade superfusion bioassay (67). DNICs and/or *S*-nitrosothiols also seem unable to account for the relaxing effect of MDRF, which was not affected by interventions aimed at the inactivation (*S*-nitrosothiols) or trapping (DNICs) of these NO carriers. Moreover, despite the use of sensitive assays, neither *S*-nitrosothiols nor DNICs could be detected in the effluate from IL-1β-treated smooth muscle cells. Although the possibility that small amounts of DNICs or *S*-nitrosothiols are released from these cells cannot be entirely ruled out, it seems unlikely that they make a major contribution to the vasorelaxant effect of MDRF.

Another candidate for MDRF was the adduct HOArg-NO. At physiological pH, this substance is spontaneously generated upon reaction of HOArg with NO (12, 13). Like MDRF, the relaxing effect of the HOArg-NO adduct is abolished in the presence of oxyhemoglobin (12), and its biological half-life of 10–15 min at physiological pH and ambient temperature (12, 13) closely matches that of MDRF, which was estimated to be ≈12 min under these conditions. The pharmacological profiles of MDRF and the HOArg-NO adduct also appear to be indistinguishable, further supporting the notion that these two substances may in fact be the same.

Unfortunately, the insensitivity of the HPLC/UV detection method precluded a direct determination of the concentration of the HOArg-NO adduct in the effluate from the column of IL-1β-stimulated smooth muscle cells. Moreover, collecting enough material from the HPLC fraction corresponding to the HOArg-NO adduct to elicit an appreciable relaxant response also proved impossible. Since lyophilization destroyed its biological activity, we would have had to inject ≈100 ml of the effluate onto the analytical HPLC column. However, due to the high ionic strength of the sample (Krebs–Henseleit solution) and other technical reasons, the sample volume could not exceed 100 μl.

Therefore, the concentration of its precursor (and decomposition product), HOArg, was determined in the conditioned medium of cultured smooth muscle cells that had been exposed to IL-1β for 20 h. There was indeed a N^G-nitro-L-arginine-sensitive accumulation of HOArg in the supernatant, but not

in cell lysates, the concentration of which (≥5 μM) clearly exceeded that (≥1 μM) required for the formation of the HOArg-NO adduct under bioassay conditions (12). Significant amounts of HOArg (≥1 μM) were also detected in the supernatant of IL-1β-stimulated smooth muscle cells after a short-term incubation in Krebs–Henseleit solution. Most importantly, however, the effluate of these cells contained up to 100 pmol of HOArg per ml. In view of the rapid decomposition of the HOArg-NO adduct upon derivatization, it cannot be stated with certainty whether this amount of HOArg included or excluded the HOArg-NO adduct. If it indeed reflects the breakdown of the HOArg-NO adduct to HOArg (≈50%) upon derivatization of the sample, then the concentration of the adduct in the effluate may have been as high as 200 pmol/ml.

In this context, it is important to consider the rapid reaction of NO with HOArg (≤1 s) under bioassay conditions and the potent vasorelaxing activity of the resulting HOArg-NO adduct. Indeed, with the exception of some hemoproteins and superoxide anions, HOArg appears to be the only naturally occurring substance that can directly react with NO at neutral pH. Thiols, for example, can only be nitrosylated at acidic pH, and this may explain why HOArg-NO adduct formation occurs irrespective of the presence of equimolar concentrations of L-cysteine (unpublished observation). Thus, the aforementioned experimental evidence, albeit largely indirect, suggests that the HOArg-NO adduct and MDRF are probably identical.

Another interesting aspect of the present study was that almost 10% of the L-arginine consumed by the NO synthase in IL-1β-stimulated smooth muscle cells appeared as HOArg in the extracellular space. In fact, the ratio of L-citrulline to HOArg formation seemed to be fairly constant at 10:1. Similar ratios were also obtained with crude NO synthase preparations, e.g., from freshly isolated porcine aortic endothelial cells (12% HOArg) and from lipopolysaccharide-stimulated rat alveolar macrophages (6% HOArg; ref. 14). Moreover, an accumulation of considerable amounts of HOArg (≥17%) has been described in the conditioned medium of lipopolysaccharide/interferon γ-stimulated EMT-6 adenocarcinoma cells (15), and small amounts of HOArg (≤2%) were also detected upon incubation of the purified neuronal NO synthase with L-arginine (16). Although it is unclear at present as to how HOArg leaves the cell, two reasons most likely account for the accumulation of HOArg in the conditioned medium of IL-1β-stimulated smooth muscle cells: (*i*) the 2-fold higher K_m value of the inducible NO synthase for HOArg (17) favors its displacement from the active site by L-arginine and (*ii*) HOArg and L-arginine compete for the same amino acid carrier (system y^+; ref. 18), so that HOArg reuptake may only be facilitated if the extracellular concentration of L-arginine falls below a critical threshold. The finding that no HOArg was detectable in the cell lysate may be explained by the fact that any HOArg remaining in the cell can be metabolized to NO and L-citrulline by enzymes other than NO synthase, such as cytochrome P450 monooxygenases (18).

In cytokine-stimulated cells, such as smooth muscle cells, macrophages, or neutrophils, where the expression of the inducible isoform of NO synthase leads to a continuous production of NO, HOArg is likely to be released into the extracellular space and to react with the concomitantly released NO, yielding the HOArg-NO adduct. Once formed this intercellular NO carrier may have important paracrine effects that may extend well beyond its potent vasorelaxant activity.

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