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Immunology

Mouse peritoneal macrophages release leukotriene C in response to a phagocytic stimulus

(slow reacting substance/prostaglandin/arachidonic acid/phagocytosis/zymosan)

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ABSTRACT **Mouse peritoneal macrophages that had ingested zymosan particles released a polar metabolite of arachidonic acid possessing slow-reacting substance activity in the guinea pig ileum assay. The metabolite was purified by solvent extraction, Sephadex G-25 column chromatography, and high-pressure reverse-phase liquid chromatography. The purified metabolite absorbed light at 280 nm and contained a free amino group. When macrophages were preincubated overnight with [^{3}H]arachidonic acid, [^{35}S]cysteine, or [^{14}C]glutamic acid, each radiolabel was incorporated into the compound. Direct amino acid analysis revealed glycine, glutamic acid, and cysteine at molar ratios of 0.97:1.00:0.82. The above data were consistent with the structure of leukotriene C, an adduct of arachidonic acid and glutathione. Quantification of the leukotriene C based on incorporation of [^{3}H]arachidonic acid or amino acid analysis indicated that 6×10^7 macrophages (3.6 mg of cell protein) released 7.5 nmol after a maximal phagocytic stimulus. The purified leukotriene C had a slow reacting substance activity of 11,500 units/nmol (1 unit has the activity of 5 ng of histamine in a guinea pig ileum contraction assay).**

Recent studies in this and other laboratories have shown that macrophages contain large amounts of esterified arachidonic acid (20:4) in their phospholipids (1, 2) and that up to 50% of this 20:4 is released in the form of oxygenated metabolites in response to a phagocytic stimulus (1). These metabolites have been identified as prostaglandins and hydroxyicosatetraenoic acids (1). More detailed recovery experiments, however, revealed that significant quantities of a more polar species were being lost in previous fractionation procedures. The solubility properties and behavior of this material on thin-layer chromatography subsequently led us to hypothesize that this metabolite was leukotriene C (LTC), a recently described slow reacting substance (SRS) isolated by Murphy *et al.* (3) from a mouse mastocytoma. This compound has been identified as a hydroxyicosatetraenoic acid containing glutathione as a substituent in a thioether linkage at C6 (4).

This communication will document that LTC is a major secretory product of resident mouse peritoneal macrophages and report on its induction, purification, and biological and chemical properties.

MATERIALS AND METHODS

Macrophage Cultures. Primary cultures of peritoneal macrophages were established from resident cells of female Swiss Webster mice (Taconic Farms, Germantown, NY) weighing 25–30 g as described (5). Peritoneal cells (6×10^6) in alpha minimal essential medium (α-MEM, GIBCO) containing 10% fetal calf serum were added to 35-mm-diameter plastic culture dishes. After 2 hr at 37°C in 95% air/5% CO_2, cultures were washed three times in α-MEM to remove nonadherent cells and incubated overnight (16 hr) in fresh medium plus 10% fetal calf serum containing radiolabeled precursors as described below.

Preparation of Unopsonized Zymosan. Zymosan was purchased from ICN, and stock solutions in α-MEM were prepared as described (6).

Synthesis of Radiolabeled LTC by Macrophage Cultures. RPMI medium deficient in cysteine or glutamic acid and glutathione was prepared using an RPMI Select-Amine Kit (GIBCO) with replacement of RPMI vitamins with minimal essential medium vitamins. To obtain LTC labeled in the cysteine moiety, 1 ml of cysteine-deficient RPMI plus 10% fetal calf serum containing [^{35}S]cysteine (specific activity 284 mCi/mmol, Amersham/Searle; 1 Ci = 3.7×10^{10} becquerels) at 14.6 μCi/ml was added to each culture dish prior to the 16-hr incubation period. Labeling with [^{14}C]glutamic acid was similarly performed, using glutamic acid-deficient RPMI and L-[1,2,3,4,5-^{14}C]glutamic acid (specific activity 267 mCi/mmol, New England Nuclear) at 1.58 μCi/ml.

For all other experiments, LTC was labeled in the arachidonic acid moiety by the addition of 1 ml of α-MEM plus 10% fetal calf serum containing 0.5 μCi [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid ([^{3}H]20:4, specific activity 62.2 Ci/mmol, New England Nuclear) per 35-mm culture dish.

At the end of the 16-hr incubation period, cells were washed twice in α-MEM and fresh α-MEM (no serum) containing zymosan at 160 μg/ml was added. After incubation for 1 hr at 37°C in 5% CO_2 to allow phagocytosis to occur, the medium was removed and LTC was extracted.

The cell monolayers were washed twice with phosphate-buffered saline, overlaid with 0.05% Triton X-100 (Rohm and Haas, Philadelphia, PA), and the dishes were scraped. Cell protein was determined by the method of Lowry *et al.* (7). Macrophages isolated from 6×10^6 peritoneal cells contained approximately 150 μg of protein.

Purification of LTC

Extraction of LTC. Quantities given refer to 1 ml of medium. The medium was acidified to pH 3 with 10 μl of 85% (wt/wt) formic acid. Then 3.75 ml of chloroform/methanol (1:2, vol/vol), 1.25 ml of chloroform, and 1.25 ml of water were added sequentially, with mixing after each addition. After centrifugation at $1000 \times g$ for 15 min, the lower phase was removed and washed with 4 ml of fresh upper phase (prepared by sequential addition of 3.75 ml of chloroform/methanol (1:2, vol/vol), 1.25 ml of chloroform, and 1.25 ml of water to 1 ml of phosphate-buffered saline acidified to pH 3 with formic acid

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Abbreviations: LTC, leukotriene C; 20:4, arachidonic acid; α-MEM, alpha minimal essential medium; SRS, slow reacting substance; HPLC, high pressure liquid chromatography.

and removal of the lower phase by centrifugation). The upper phases were combined and extracted with 12 ml of chloroform/ethanol (1:1, vol/vol) and 4 ml of chloroform/ethanol (2:1, vol/vol). The chloroform/ethanol extracts were combined and concentrated under reduced pressure. The extraction was performed at 4°C under a nitrogen atmosphere. Solvents contained 0.005% butylated hydroxytoluene (Sigma), an antioxidant.

Sephadex G-25 Column Chromatography of LTC. The concentrated chloroform/ethanol extract was evaporated to near dryness, dissolved in 0.5 ml of 50% (vol/vol) ethanol/water, and placed on a column of Sephadex G-25 (Pharmacia). The column (2 cm × 21 cm) was eluted at room temperature with 50% (vol/vol) ethanol/water containing 0.005% butylated hydroxytoluene at a flow rate of 18 ml/hr. Fractions (1.7 ml) were collected. LTC, which eluted in fractions 16–30, was concentrated under reduced pressure.

High-Pressure Liquid Chromatography (HPLC) of LTC. Reverse-phase HPLC purification of LTC was performed on a column (4.6 mm × 25 cm) of Lichrosorb C-8 (Altex, Rainin Instruments, Brighton, MA). The column was eluted with 17 ml of buffer 1 (0.123 M pyridine/0.4 M formic acid/0.001% thiodiglycol, pH 3.25) followed by a linear gradient between buffer 1 and buffer 2 [0.123 M pyridine/0.4 M formic acid/0.001% thiodiglycol/40% (vol/vol) propanol/60% (vol/vol) water, pH 3.70] totaling 90 ml. The flow rate was 34 ml/hr. Compounds containing free amino groups were detected after reaction with fluorescamine (8, 9) with a Gilson Spectro Glo fluorometer, using an on-line sampling system described by Böhlen *et al.* (10). The column effluent was simultaneously monitored for absorbance at 280 nm, using a Uvicord spectrometer (LKB). Fractions (1.4 ml) were collected and the radioactivities of samples were measured by using an LKB 1210 Ultrobeta scintillation counter. Counting efficiencies were 40% for ^{3}H, 77% for ^{14}C, and 90% for ^{35}S.

Amino Acid Analysis. Fractions containing purified LTC from HPLC were dried under reduced pressure and hydrolyzed in evacuated ampoules in 6 M HCl for 20 hr at 110°C. Amino acid analysis was performed on a Durrum D-500 automatic amino acid analyzer by previously described methods (11, 12).

Bioassay of LTC. The bioassay for SRS activity was performed by using an isolated guinea pig ileum in Tyrode's solution in the presence of atropine (1 μM) and pyrilamine maleate (1 μM), by the method of Chakravarty (13). One unit (U) of SRS activity was arbitrarily defined as that amount producing a contraction equal in amplitude to that elicited by 5 ng of histamine. Fractions from HPLC were evaporated under reduced pressure and dissolved in Tyrode's solution for testing. Contractions induced by purified LTC preparations were inhibited by the specific SRS antagonist FPL 55712 (Fisons Pharmaceuticals, Loughborough, England) (14) at drug concentrations of 0.01 μg/ml.

RESULTS

Mouse resident peritoneal macrophages were purified by adherence and labeled overnight (16 hr) with [^{3}H]20:4. Unopsonized zymosan (160 μg/ml) was then added to the cultures, and the cells were incubated for 1 hr at 37°C in 5% CO_2 to allow phagocytosis to occur. LTC was purified from the culture medium.

The outstanding feature of the purification scheme for LTC described in *Materials and Methods* is the extraction, which allows a significant initial purification of LTC from other medium components under very mild conditions. Extraction of [^{3}H]20:4, [^{3}H]prostaglandin E_2, and [^{3}H]valine standards in α-MEM showed that 1%, 25%, and 25% of these compounds, respectively, remained as contaminants in the final chloroform/ethanol phases. Because most cell neutral lipids and phospholipids partition into the lower phase in the first extraction step (15), procedures such as base hydrolysis and silicic acid chromatography, which have been used to purify LTC in the past (3), were not necessary. Only desalting and the removal of small molecules such as amino acids and simple sugars by Sephadex G-25 chromatography was necessary prior to HPLC.

Fig. 1 shows a typical HPLC elution profile of LTC produced by macrophages prelabeled with [^{3}H]20:4. Profiles of radioactivity, bioactivity, UV absorbance, and relative fluorescence after reaction with fluorescamine are shown. At a retention time of 165 min, a ^{3}H-labeled substance was eluted from the column that was active in the ileum assay, absorbed light at 280 nm, and possessed a free amino group as indicated by the fluorescamine detection system. No such material was found in medium from macrophages not challenged with zymosan. Two other major ^{3}H-containing peaks were obtained from zymosan-treated cultures, but not from control cultures (no zymosan). The identity of the first of these (retention time 130 min) is unknown. The second had an identical retention time as [^{3}H]-prostaglandin E_2 standard; however, it demonstrated no bioactivity in the ileum assay. As a result, only one bioactive peak, corresponding to the material eluting at 165 min, was obtained.

We concluded that the material obtained was a 20:4 me-

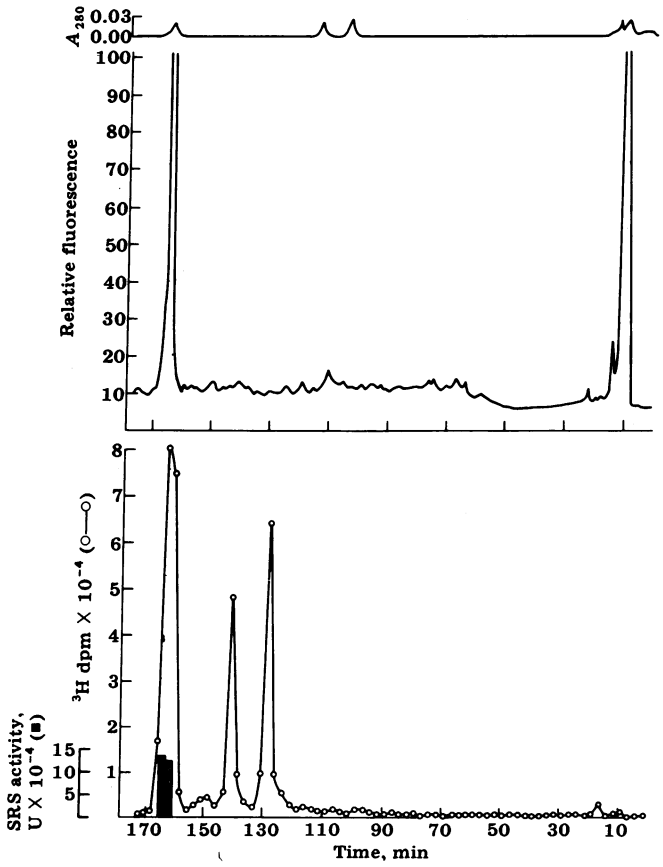

FIG. 1. HPLC elution profiles of LTC purified from macrophage medium. Twenty-four macrophage cultures were prelabeled with [^{3}H]20:4 as described. After zymosan challenge, the medium was subjected to extraction, Sephadex G-25 chromatography, and HPLC. The HPLC eluate was monitored for ^{3}H (○—○), UV absorbance at 280 nm (—), fluorescamine-positive material (—), and SRS bioactivity (■).

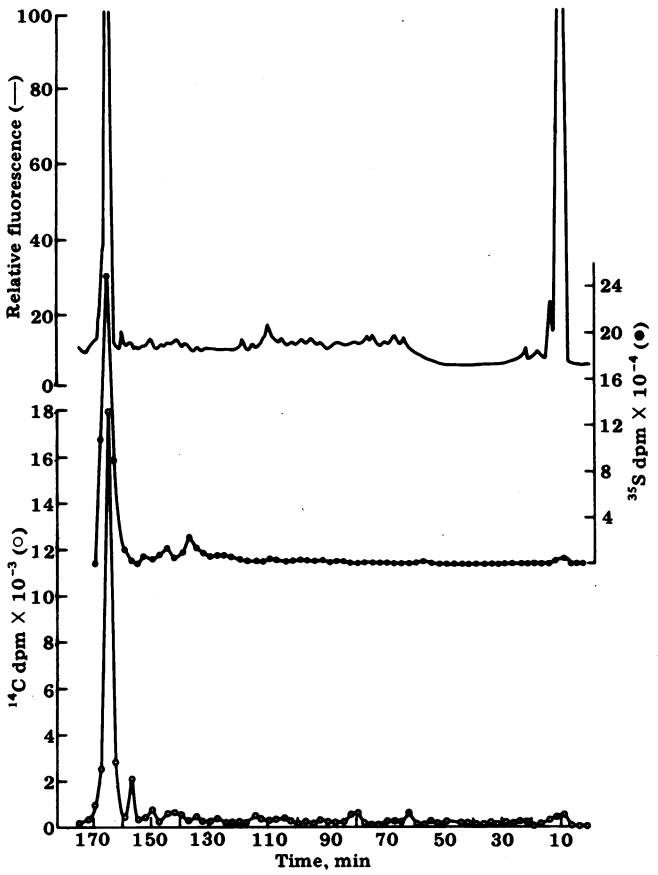

FIG. 2. Labeling of LTC with radioactive amino acid precursors. LTC was purified from macrophage cultures prelabeled overnight with radioactive amino acids and challenged with zymosan. Either 32 cultures ([^{35}S]cysteine) or 24 cultures ([^{14}C]glutamic acid) were used. The eluates were monitored for fluorescamine-positive material (—) and ^{14}C (○—○) or ^{35}S (●—●).

tabolite having SRS activity, at least one free amino group, and a chromophore that absorbed at 280 nm. All of these properties were consistent with the structure of LTC. Further evidence was obtained from studies in which macrophages were prelabeled with radioactive amino acids and then challenged with zymosan. Fig. 2 shows HPLC elution profiles from two separate experiments in which macrophages were cultured overnight in the presence of [^{35}S]cysteine or [^{14}C]glutamic acid. In each experiment, major peaks of radioactivity appeared at a retention time of 165 min that coincided with a peak in the fluorescamine monitoring system. This provided evidence that cysteine and glutamic acid or metabolic products of these amino acids were present in the fluorescamine-positive peak.

More direct evidence for the amino acid composition of the substance was obtained by analysis of an acid hydrolysate of material from fractions eluting at 165 min. These results are presented in Table 1. When hydrolysis in HCl was performed under vacuum glycine, glutamic acid, and cysteine (determined as half-cystine) were obtained at molar ratios of 0.97:1.00:0.82. In a second experiment, when hydrolysis was not performed under vacuum, glycine, glutamic acid, and cysteic acid were recovered at ratios of 0.99:1.00:0.84. Thus, all components of the glutathione substituent were obtained in ratios consistent with the structure of LTC.

Table 1. Amino acid analysis of LTC

Amino acid	Relative amounts	
	Exp. 1	Exp. 2
Glycine	0.97	0.99
Glutamic acid	1.00	1.00
Half-cystine	0.82	—
Cysteic acid	—	0.84

LTC was purified from 24 macrophage cultures (3.6 mg of cell protein) and hydrolyzed in 6 M HCl. Exp. 1, hydrolysis performed under vacuum; Exp. 2, hydrolysis not performed under vacuum. Values are expressed relative to glutamic acid.

LTC was extracted and purified from the medium derived from macrophages (3.6 mg of cell protein) prelabeled with [^{3}H]20:4 and challenged with a maximal phagocytic load of zymosan (160 μg/ml). The material recovered in HPLC fractions that eluted at 165 min contained 155,000 dpm of ^{3}H. On the basis of the specific activity of [^{3}H]20:4 in the cell phospholipid [5.4 × 10^{4} dpm/nmol (1)], this amount of radiolabel corresponded to 2.9 nmol of LTC. Amino acid analysis of a portion of this sample indicated the presence of 3.7 nmol of LTC in the entire sample. Fig. 3 shows the histamine dose–response determination and bioassay of a sample of this LTC containing 575 dpm (10.7 pmol). The assay results indicated that LTC possessed a bioactivity of 11,500 U/nmol.

When HPLC-purified ^{3}H-labeled LTC was added to fresh α-MEM and repurified, the recovery of radioactivity after the extraction procedure was 70%. After the final HPLC purification, recovery of radiolabel was 40%, and the recovery of bioactivity was 34%. On the basis of these data, the 3 nmol of LTC recovered from macrophages after a maximal zymosan stimulus corresponded to a total production of approximately 7.5 nmol or 2.1 nmol/mg of cell protein. The bioactivity recovered corresponded to 24,150 U/mg of cell protein. The total ^{3}H label released by the cells totalled 930,000 dpm/mg of cell protein. Therefore, LTC composed 10–15% of the 20:4 metabolites released by the cells under these conditions.

DISCUSSION

SRS was discovered in the perfusate from a guinea pig lung after treatment with cobra venom (16). Later work demonstrated that SRS was also generated in passively or actively sensitized lungs after challenge with antigen (17, 18). Other investigators have used passive sensitization and antigen challenge of rat peritoneal cells *in vivo* as a source of SRS (19). The generation of SRS has been associated with conditions involving immediate hypersensitivity, and the IgE-mast cell-initiated generation of SRS in the rat peritoneal cavity has been reported (20). However, attempts to elicit SRS production by antigenic

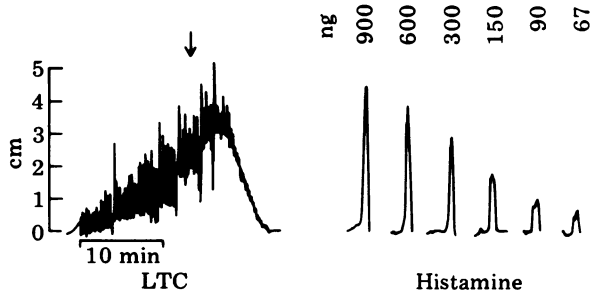

FIG. 3. Bioassay of LTC. LTC was purified from medium from 24 macrophage cultures (3.6 mg of cell protein) prelabeled with [^{3}H]20:4 and challenged with zymosan. Bioassay was performed on an isolated guinea pig ileum in Tyrode's solution containing 1 μM atropine. After calibration with histamine, pyrilamine maleate (1 μM) was added, and LTC was tested. Characteristic responses are shown for various doses of histamine as well as the response to 11 pmol of LTC. Arrow indicates washout. Tracing progresses from right to left.

challenge of purified rat mast cells have not been successful (21). Depletion of mast cells from the rat peritoneal cavity failed to prevent SRS release after treatment with hyperimmune serum and antigen, although histamine release after stimulation with IgE plus antigen was markedly inhibited (22). Furthermore, a class of IgG has been shown to mediate release of SRS but not histamine from the rat peritoneal cavity (23). Other cell types, therefore, including macrophages (24), have been suggested as possible sources of SRS.

In this paper, we present evidence that, when triggered with an appropriate stimulus, mouse peritoneal macrophages synthesize and release large amounts of a highly biologically active SRS having properties consistent with the structure of LTC. That this occurs is not surprising when one considers the high 20:4 content of macrophage phospholipids (25%) and the extensive release (40–50%) of this 20:4 as oxygenated metabolites after a phagocytic challenge. As a result of this and other work (1), it is now possible to account for all of the [^{3}H]20:4 released by macrophages in response to a phagocytic stimulus. Approximately 51% can be identified as prostaglandin E, 16% as 6-oxoprostaglandin $F_{1\alpha}$, 10–15% as LTC, and the remainder as hydroxyicosatetraenoic acids. We have designated the bioactive compound isolated from mouse macrophages as LTC, because our evidence is consistent with the structure of that compound. It is important to note, however, that we have no information concerning the final structure of the incorporated 20:4 moiety or its linkage to the peptide portion of the molecule. Therefore we cannot know with absolute certainty that the structure of macrophage SRS is identical to that of LTC as described by Hammarström *et al.*

If the macrophage SRS is in fact LTC, these findings raise some important questions concerning the role of LTC in immediate hypersensitivity reactions as well as the identity of LTC with SRS from more traditional sources. Attempts have been made in the past to extract SRS from guinea pig lung and rat peritoneum into organic solvents from acidified aqueous media (25, 26). In each case, the SRS activity was found to partition into the organic phase; however, only a fraction of the activity could be recovered (30–50%). We confirmed these findings in our extraction system, using a lyophilized preparation of SRS having a known bioactivity, obtained from sensitized guinea pig lung perfused after antigen stimulation (Fisons Pharmaceuticals). Bioactivity was found only in the lower phase of the first extraction step, and only 30% of the original activity was recovered. In contrast, LTC partitioned into the upper phase in this step of the extraction, and recovery of activity was 75%. These findings indicate differences in the structures of LTC and SRS from sensitized guinea pig lung. That SRS is in reality a family of compounds, or that different compounds are produced by different cells or organs, has been suggested in the past (27, 28). These observations are consistent with such possibilities.

A second question that arises concerns the nature of the trigger used for SRS generation. Binding of IgE to macrophages followed by treatment with antigen is not well recognized as a stimulus for these cells. Therefore, if macrophages are the source of SRS in systems in which IgE and antigen are used as the trigger, the exact nature of the stimulus recognized by the macrophage is of interest. One possibility is that immune complexes of IgE and antigen or other particulate matter are present and serve to trigger the release of 20:4 metabolites. Another possibility is that mast cells and macrophages interact in some way leading to macrophage stimulation, as has been suggested for the production of platelet-activating factor (29). The possible roles of different cell types and various stimuli in LTC production remain to be elucidated.

In their purification by HPLC, Murphy *et al.* (3) reported the appearance of two bioactive compounds, which they designated LTC-1 and LTC-2. LTC-1 was the predominant compound, and all further structural work was performed on it. The exact difference between LTC-1 and LTC-2 remains obscure. In our purification scheme, only one peak of bioactivity was obtained with consistency. In several samples, a second, small, fluorescamine-positive, radiolabeled peak appeared at a retention time of 168 min. The presence of this peak seemed to correlate with overexposure of samples to heat or air. It is therefore possible that this component was a degradation or oxidation product of LTC.

The bioactivity of LTC-1 on a molar basis has been reported to be approximately two orders of magnitude higher than that of histamine (4). Our results indicate an even higher potency (500–1000 times higher than histamine). The reason for this discrepancy is unclear. It may relate to subtle differences in assay conditions, but it may also relate to actual differences in the molecules themselves.

Macrophages are recognized as important mediators in chronic inflammatory responses. They are found in large numbers in sites of inflammation, and their longevity makes them well suited for this role (30). Their involvement in immediate hypersensitivity reactions, however, has not been well studied. If LTC is indeed an SRS produced in immediate hypersensitivity states, macrophages may prove to be important mediators of such responses. On the other hand, one must also consider a role for LTC in more chronic inflammatory states. In sites of inflammation, macrophages are constantly presented with stimuli which have been shown to cause the release of 20:4 metabolites (31). Therefore, the possibility for the release of LTC in such sites most certainly exists. It is clear that much work remains to be done to clarify the physiologic and pathologic role of this intriguing molecule.

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