THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 45, Issue of November 8, pp. 28077–28081, 1996

Printed in U.S.A.

Cystatins Up-regulate Nitric Oxide Release from Interferon-γ-activated Mouse Peritoneal Macrophages*

(Received for publication, August 10, 1995, and in revised form, March 19, 1996)

Ludovic Verdot‡§¶, Gilles Lalmanach‡, Vincent Vercruysse§, Susanne Hartmann‖, Richard Lucius‖, Johan Hoebeke‡, Francis Gauthier‡, and Bernard Vray§****

From the §Laboratoire d'Immunologie Expérimentale (CP 615), Faculté de Médecine, Université Libre de Bruxelles, B1070 Brussels, Belgium, the ‡Laboratoire d'Enzymologie et de Chimie des Protéines, Université François Rabelais, F37032 Tours, France, and ‖Molecular Parasitology (Institut of Biology), Humboldt-University Berlin, Berlin, Germany

Up-regulation of nitric oxide (NO) production by activated murine macrophages was observed during infection by *Trypanosoma cruzi*, the etiological agent of Chagas' disease. Cell infection by *T. cruzi* depends at least in part on cruzipain, a membrane-associated papain-related proteinase which is sensitive to inhibition by synthetic inhibitors of cysteine proteinases. Using the natural cysteine proteinase inhibitor chicken cystatin, a representative member of cystatin family 2, to investigate the effect of cruzipain on macrophage infection and NO release, we found that the inhibitor alone up-regulated NO release from interferon-γ-activated macrophages. A 12-fold increase in NO production was observed in the presence of 1 μM chicken cystatin. This overproduction was concentration-dependent and could be detected at concentrations as low as 10 nM and remained in the presence of polymyxin B. Representative members of the other cystatin families, *i.e.* stefin B (family 1), T-kininogen, and its inhibitory domains (family 3), were also able to enhance NO production from interferon-γ-activated macrophages. Neither E64, an irreversible inhibitor of cysteine proteinases, nor inhibitors of aspartyl and serine proteinases (aprotinin, pepstatin, and soybean trypsin inhibitor) enhanced NO production. Upon complexation with saturating amounts of reduced-alkylated papain, cystatins still remained active in increasing NO production, suggesting that the cystatin inhibitory site was not involved in the mechanism.

The results demonstrate that members of all 3 cystatin families share another common property unrelated to their function of cysteine proteinase inhibitors, *i.e.* up-regulation of NO production, which biological significance remains to be elucidated.

Nitric oxide (NO)[1] is a multipotent physiologic molecule detected in the immune, neuronal, and vascular systems and in many other tissues (1). NO is synthesized by a wide variety of cell types from L-arginine by NO synthases (NOS), three distinct isoforms of which have now been identified (2, 3). Two isoforms are constitutive and Ca^{2+}-calmodulin dependent: the first is membrane-bound, and was initially discovered in endothelial cells; the second is soluble and was first identified in neurons. The third isoform is an inducible Ca^{2+}-independent NOS first discovered in murine macrophages and induced by appropriate stimulation with cytokines (4–7).

Among other cells, macrophages allow the intracellular multiplication of *Trypanosoma cruzi*, the etiological agent of Chagas' disease, a major public health problem in South and Central America (8). A close relationship has been demonstrated between NO production in activated murine macrophages and *T. cruzi* infection (9). The parasite modulates the NO production by cytokine and LPS-activated macrophages (10, 11). Processes involved in macrophage infection with *T. cruzi* are extensively studied (12). The membrane cysteine proteinase cruzipain of *T. cruzi*, which corresponds to the major antigen gp 57/51 (13–17), has been shown to be involved in macrophage infection during the first step of the parasite-macrophage recognition using anti-cruzipain antibodies (18, 19) and synthetic irreversible inhibitors of the cysteine proteinases (20, 21).

Cysteine proteinases are regulated by tight-binding and reversible natural inhibitors that belong to the cystatin superfamily. Heterologous cystatins from chicken, rat, and human bind and inhibit cruzipain (22).[2] Thus, to determine whether natural inhibitors of cysteine proteinases could modulate the infection of macrophages by *T. cruzi* and NO synthesis, preliminary assays have been performed in the presence of chicken cystatin, the best characterized inhibitor of cysteine proteinases (23–25). These experiments led to the unexpected observation that chicken cystatin stimulated NO production by IFN-γ-activated macrophages.

To substantiate this observation, we examined the capacity of representative members of the three main cystatin families (stefins, cystatins, and kininogens) to modulate NO release of IFN-γ-activated murine macrophages. Moreover, we investigated whether the proteinase inhibitory site of cystatins is involved in this process.

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 medium, minimal essential medium (MEM) supplemented with 25 mM HEPES, 2 mM glutamine, fetal calf serum, penicillin, streptomycin, polymyxin B sulfate, and Hanks' balanced salt solution were from Life Technologies, Inc. MEM lacks nitrate and was thus used to measure nitrite and nitrate production. MEM was supplemented with L-arginine to reach the same concentration (240 mg/liter) as in RPMI 1640 medium. α_2-Macroglobulin, aprotinin, bradykinin, papain, and pepstatin were from Boehringer Mannheim (Mannheim,

* This work was supported in part by a grant of Action de Recherche Concertée, U.L.B. 1991, 1994, and 1996. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of a grant from the Region Centre (France).

** To whom correspondence should be addressed: Laboratoire d'Immunologie Expérimentale (CP 615), Faculté de Médecine, Université Libre de Bruxelles, 808 route de Lennik, B-1070 Brussels, Belgium. Tel.: 32-2-555-62-60; Fax: 32-2-555-61-28; E-mail: bvray@med.ulb.ac.be.

[1] The abbreviations used are: NO, nitric oxide; MEM, minimal essential medium; IFN-γ, interferon gamma; LPS, lipopolysaccharide; MPM, mouse peritoneal macrophages; L-NMMA, N^{ω}-monomethyl-L-arginine; NOS, nitric oxide synthase.

[2] Serveau, S., Lalmanach, G., Juliano, M. A., Scharfstein, J., Juliano, L., and Gauthier, F. (1996) *Biochem. J.* **313,** 951–956.

Germany). Human stefin B (cystatin B) was from BioAss (Gießen, Germany). E64, soybean trypsin inhibitor, sulfanilamide, naphthylethylene diamine dichloride, phosphoric acid, nitrate reductase, NADPH, FAD, and L-lactic dehydrogenase, and sodium pyruvate were from Sigma. Chicken cystatin, L-NMMA were from Calbiochem Co. (La Jolla, CA). The human macrophage THP1 cell line was kindly provided by Prof. De Baetselier (Unit of Cellular Immunology, Vrije Universiteit Brussel, Sint Genesius Rode, Belgium). LPS-free mouse recombinant IFN-γ was kindly provided by Prof. A. Billiau and Dr. H. Herremans (Katholieke Universiteit Leuven, Leuven, Belgium).

Murine Peritoneal Macrophage Culture—Male BALB/c mice, 6–8-weeks old (Bantin & Kingman Universal Ltd., United Kingdom), were killed by ether inhalation. MPM were harvested by washing twice the peritoneal cavities with ice-cold Hanks' balanced salt solution without Ca^{2+} and Mg^{2+}. MPM were centrifuged at 400 × *g* for 10 min at 4 °C. Supernatant was discarded and 1 ml of distilled sterile water was added for 30 s to lyse red cells. MPM were immediately resuspended in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} and centrifuged as above. The resulting pellet was resuspended in culture medium consisting of RPMI 1640 medium supplemented with 25 mM HEPES, 2 mM glutamine, 10% fetal calf serum (mycoplasma free and endotoxin concentration less than 27.5 pg/ml) and containing penicillin (100 IU/ml) and streptomycin (100 μg/ml). MPM were then allowed to adhere (1.5×10^5 MPM/well) in 96-well microplates (NUNC, Roskilde, Denmark) for 2 h at 37 °C in a 5% CO_2 water-saturated atmosphere in cell culture medium. Nonadherent cells were removed by washing with prewarmed culture medium before adding appropriate solution diluted in culture medium.

T. cruzi Infection—*T. cruzi* trypomastigotes (Tehuantepec strain) were obtained from the blood of X-irradiated infected F344 Fischer rats (Iffa Credo, Belgium) by ion-exchange chromatography on DEAE-cellulose (Whatman DE52) equilibrated with phosphate/saline/glucose buffer at pH 7.4. Trypomastigotes were centrifuged (15 min, 1800 × *g*, 4 °C) and resuspended in RPMI 1640 culture medium before infection of macrophages.

Murine 2C11-12N and RAW 264.7 Macrophages Cell Line Cultures—Murine hybridoma macrophage 2C11-12N and RAW 264.7 cells were cultured as described above in Falcon flasks (N 3024, Becton Dickinson, Franklin Lakes, NJ). Before experiments, cells were washed once with ice-cold Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} before incubating them in the same ice-cold solution for 5 min at room temperature. Cells were then detached by gently shaking the flasks. Cell suspensions were centrifuged and macrophages cultured (1.5×10^5 macrophages/well) as described above.

Rat T-kininogen and Its D1–2 and D3 Cystatin-like Domains—Rat T-kininogen and its cystatin-like domains D1–2 and D3 were purified as described previously (26).

Saturation of Cystatin Inhibitory Sites by Carboxymethylated Papain—One mg of papain was activated for 5 min in 0.1 M phosphate buffer, pH 6.8, 1 mM EDTA, 0.1% Brij 35, 2 mM dithiothreitol before adding iodoacetic acid. After 1 h of incubation at 37 °C in the dark, the excess of alkylating reagent was removed by desalting using a PD10 column (Pharmacia, Uppsala, Sweden). The completion of inactivation was checked by the complete loss of enzymatic activity against the fluorogenic substrate Z-Phe-Arg-NHMec.

Cystatin-carboxymethylated papain complexes were made mixing chicken cystatin, stefin, T-kininogen, and its cystatin-like domains with a large excess of papain (10 μM final) at 37 °C during 45 min. Saturation of inhibitory sites was checked by measuring the residual activity of native papain on Z-Phe-Arg-NHMec substrate in the presence or not of saturated inhibitor.

Determination of NO Concentration—NO production was evaluated by measuring nitrite, its stable degradation product, by the Griess' reaction (27). 100 μl of each culture supernatant were added to 100 μl of the Griess solution (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2% H_3PO_4). The absorbance was measured at 540 nm in a microplate enzyme-linked immunosorbent assay reader (Titertek Multiscan MCC/340, MKII EFLAB, Finland). Sodium nitrite ($NaNO_2$) diluted in culture medium was used as a standard. The detection limit of the assay was 1.25 μM. The production of NO by unstimulated MPM was typically around the detection limit. LPS levels of all the reagents and media were tested using the colorimetric Limulus Amebocyte Lysate assay (detection limit:1 pg/ml) (Coatest endotoxin Chromogenix, Mölndal, Sweden). In some experiments, MEM that did not contain nitrate was also used to measure nitrite and nitrate. Nitrate was detected after reduction to nitrite using nitrate reductase. Total nitrite was then determined as described above. Briefly, culture supernatants were incubated for 2 h at 37 °C in the presence of 0.1 unit/ml nitrate reductase, 120 mM NADPH, and 5 mM FAD. After nitrate reduction, NADPH was oxidized with 10 units/ml L-lactic dehydrogenase and 10 mM sodium pyruvate for 30 min at 37 °C. Griess' reagent was added, and absorbance was measured as described above.

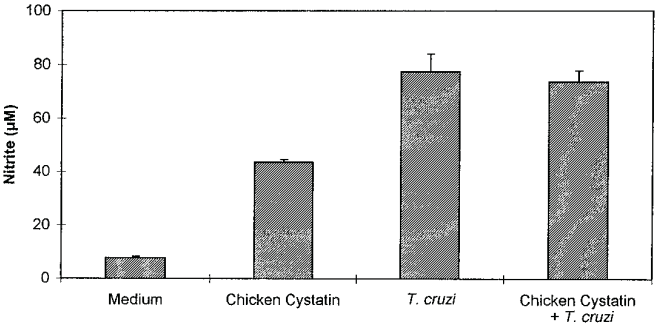

FIG. 1. **Effect of chicken cystatin on NO production by *T. cruzi* infected macrophages.** Macrophages (1.5×10^5/well) were allowed to adhere for 2 h in 96-well plates. After washing the wells with culture medium, MPM were activated with 100 IU of IFN-γ in 150 μl in medium culture for 24 h. Medium culture was then removed, and macrophages were incubated for 48 h in culture medium with: IFN-γ alone (100 IU/ml), IFN-γ (100 IU/ml) + chicken cystatin (10^{-7} M), IFN-γ (100 IU/ml) + *T. cruzi*, IFN-γ (100 IU/ml) + *T. cruzi* + chicken cystatin (10^{-7} M). The parasite-to-cell ratio was 20/1. Means ± S.D. of one experiment performed in duplicate representative of three experiments.

RESULTS

Effect of Chicken Cystatin on NO Production by T. cruzi Infected Macrophages—In order to check whether the cysteine proteinase activity of the immunodominant antigen of the *T. cruzi* cell surface was involved in the parasite-induced NO overproduction, we incubated IFN-γ-activated macrophages with *T. cruzi* trypomastigotes in the presence or absence of 0.1 μM of the cysteine proteinase inhibitor chicken cystatin, and the effects were observed on cell infectivity and NO production. Neither the percentage of infected cells (data not shown) nor NO production (Fig. 1) were modified by adding chicken cystatin. But chicken cystatin alone up-regulated the nitrite production of IFN-γ-activated macrophages (Fig. 1).

Up-regulation of the Nitrite Production by Chicken Cystatin on IFN-γ-stimulated MPM—We then precisely investigated the NO up-regulation by chicken cystatin. As shown in Fig. 2*A*, the nitrite level was significantly increased (5-fold) after 24 h of incubation when 0.1 μM chicken cystatin was added simultaneously with IFN-γ, as compared to IFN-γ alone. This up-regulation was still more pronounced (about 8-fold) after 48 h of incubation. In contrast, chicken cystatin added to unstimulated MPM was unable to induce nitrite production after 24 or 48 h of incubation. To demonstrate that the effect observed was related to the L-arginine pathway and NOS induction, we used the specific NOS inhibitor, L-NMMA. Addition of L-NMMA in the culture medium completely abolished the nitrite production (Fig. 2*A*).

Since LPS is a powerful synergistic agent of NO induction when added with IFN-γ (5), we investigated whether the low LPS content (80 pg/ml) found in 0.1 μM chicken cystatin solution could exert a synergistic effect. Preincubation of chicken cystatin with 100 IU of the LPS inhibitor polymyxin B (10 IU final concentration) resulted in a slight decrease in nitrite production, whereas the NO-inducing effect in the control experiment, consisting of 1 ng/ml LPS preincubated with 100 IU of polymyxin B (10 IU final concentration), was completly abolished by polymyxin B (Fig. 2*B*). 10 IU of polymyxin B did not modify the nitrite production of IFN-γ-activated MPM. This demonstrates that chicken cystatin is the agent inducing the increase of nitrite. Since LPS concentrations of all reagents were in the range 5–80 pg/ml, preincubation with polymyxin B

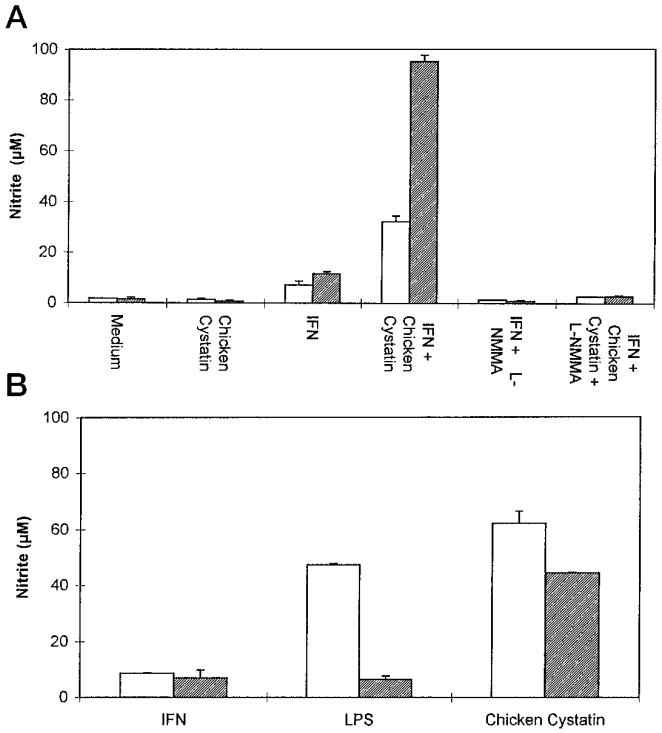

FIG. 2. **Up-regulation of nitrite release by chicken cystatin on IFN-γ-activated MPM.** Macrophages (1.5×10^5/well) were allowed to adhere for 2 h in 96-well plates. After washing the wells with culture medium, MPM were incubated in 150 μl in medium culture with the appropriate solutions. *A*, 24 h incubation (*open bars*) and 48 h incubation (*filled bars*) with culture medium, chicken cystatin alone (10^{-7} M), IFN-γ alone (100 IU/ml), IFN-γ (100 IU/ml) + L-NMMA (25.10^{-5} M), IFN-γ (100 IU/ml) + chicken cystatin (10^{-7} M), IFN-γ (100 IU/ml) + chicken cystatin (10^{-7} M) + L-NMMA (25.10^{-5} M). Means ± S.D. of one experiment performed in triplicate representative of nine experiments. *B*, macrophages were incubated for 48 h with (*open bars*) or without (*filled bars*) 2 h 100 IU of polymyxin B sulfate preincubation (10 IU final concentration) with IFN-γ alone (100 IU/ml), IFN-γ (100 IU/ml) + LPS 1 ng/ml (100 pg/ml final concentration), IFN-γ (100 IU/ml) + chicken cystatin (10^{-7} M). Means ± S.D. of one experiment performed in triplicate representative of three independent experiments.

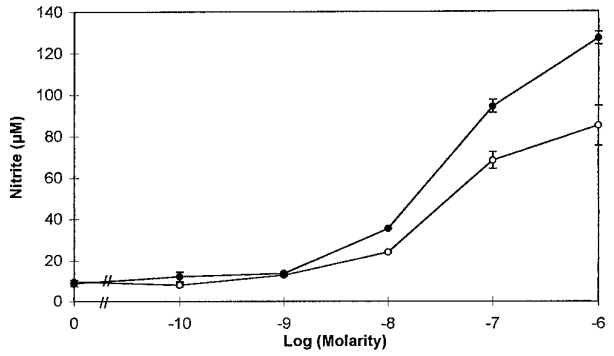

FIG. 3. **Concentration-dependence of the up-regulation of nitrite release by chicken cystatin on IFN-γ-activated MPM.** Experiment was conducted as described in the legend of Fig. 1. MPM were incubated with chicken cystatin (10^{-6} to 10^{-11} M) + IFN-γ (100 IU/ml) for 24 h incubation (*open circle*) and 48 h incubation (*black circle*). Means ± S.D. of one experiment performed in triplicate representative of five experiments.

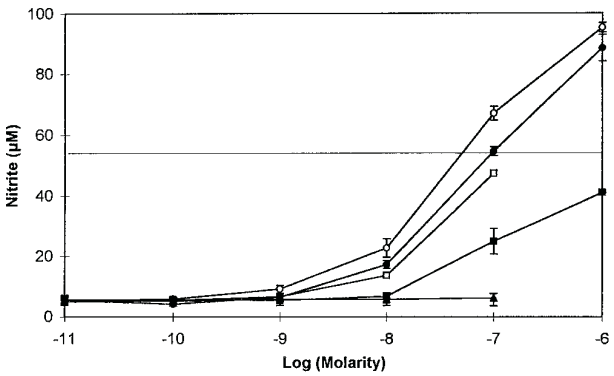

FIG. 4. **Concentration dependence of nitrite up-regulation by cystatins.** Experiment was conducted as described in the legend of Fig. 1. Human stephin B (*black square*), T-kininogen (*open square*), and its D1–2 (*open circle*) and D3 (*black circle*) cystatin-like domains were incubated in concentration ranging from 10^{-6} to 10^{-11} M with IFN-γ (100 IU/ml) 48 h of incubation. Bradikinin (*black triangle*) was incubated in the same concentration range. The *horizontal line* represents the nitrite release by chicken cystatin at a concentration of 10^{-7} M. Means ± S.D. of one experiment performed in triplicate is representative of three experiments.

as described above was a prerequisite for all subsequent experiments to prevent any synergistic effect between LPS and IFN-γ.

Synergistic Effect of the Natural Cysteine Proteinase Inhibitor Chicken Cystatin on Nitrite Production by IFN-γ-stimulated MPM Is Concentration-dependent—The enhancement of the nitrite production by chicken cystatin was concentration-dependent (Fig. 3). The effect was maximal with a concentration of 1 μM and after 48 h of incubation, an increase production of NO has been detected with a concentration of chicken cystatin as low as 10 nM (Fig. 3). Addition of 0.25 mM L-NMMA completly abolished the nitrite production (data not shown). To investigate whether the synergistic effect of chicken cystatin was not restricted to only one macrophage cell type, *i.e.* mouse peritoneal macrophages, we tested chicken cystatin on the murine 2C11-12N and the RAW 264.7 macrophage cell lines, and human THP1 macrophage cell lines. Under identical conditions, chicken cystatin also up-regulated the nitrite release by both IFN-γ-activated 2C11-12N and RAW 264.7 cell line macrophages. The synergistic effect was less pronounced than observed on MPM, since only a 1 μM concentration increased the nitrite levels up to 2-fold (data not shown). On human IFN-γ-activated human macrophage THP1 cell lines, even though NO release from human macrophages is still a matter of debate (29), no nitrite production was detected, even with high concentrations of IFN-γ. Chicken cystatin in the absence of the IFN-γ also failed to induce nitrite release (data not shown).

Up-regulation of the Nitrite Production by the Cystatin Superfamily—The cystatin superfamily of cysteine proteinase inhibitors is subdivided in three main families. Chicken cystatin belongs to family 2, whereas human stefin B and rat T-kininogen are two representative members of cystatin family 1 and 3, respectively (30). These two inhibitors as well as cystatin-like inhibitory domains D1–2 and D3 obtained from limited proteolysis of T-kininogen (26) were assayed on the nitrite production by IFN-γ-activated MPM. All cystatin superfamily members enhanced nitrite release by IFN-γ-activated MPM in a concentration-dependent manner (Fig. 4). The synergistic effect of these cystatins to the one observed with the chicken cystatin was similar except that the nitrite increasing capacity of stefin B was lower (Fig. 4). Both native T-kininogen and its D3 cystatin-like domain contain the bradykinin sequence but physiologically, this peptide is not released in significant amount from T-kininogen (31). Bradykinin was shown to stimulate tumor necrosis factor-α and interleukin-1 release from murine macrophage cell lines (32) and to increase L-arginine uptake and NO release in the vascular endothelial cells (33). Purified bradykinin was assayed on IFN-γ-activated MPM to exclude the possibility that the nitrite was not a consequence of

TABLE I

Up-regulation of nitrite and nitrate release by cystatins on IFN-γ-activated MPM in nitrate-depleted culture medium

Macrophages (1.5×10^5/well) were allowed to adhere for 2 h in 96-well plates in MEM. After washing the wells with MEM, MPM were incubated for 48 h in 150 μl in medium culture with the appropriate solution containing cystatins at 10^{-6} M. Nitrite and then nitrate reduced in nitrite were measured in culture supernatants. Means ± S.D. of one experiment performed in triplicate representative of three independent experiments.

Macrophage treatment	Nitrite	Nitrite + nitrate
	μmol	
No activation	1.03 ± 0.62	0
IFN-γ-activated MPM	35.81 ± 3.90	109.22 ± 14.45
IFN-γ-activated MPM + chicken cystatin	90.81 ± 3.72	225.10 ± 20.15
IFN-γ-activated MPM + T kininogen	113.29 ± 6.23	258.54 ± 40.38
IFN-γ-activated MPM + human stephin B	86.48 ± 7.69	228.08 ± 85.79
IFN-γ-activated MPM + D1–2	110.63 ± 5.07	282.28 ± 48.81
IFN-γ-activated MPM + D3	112.93 ± 5.26	289.07 ± 48.81

its release from both T-kininogen and its D3 cystatin-like domain. Bradykinin failed to increase nitrite release even after 48 h of incubation (Fig. 4).

In order to exclude that the cystatin effect was due to the activation of nitrate reductase, additional experiments have been performed in MEM culture medium that did not contain nitrate. Once again, the addition of cystatins to IFN-γ-activated MPM induced NO production and nitrite and nitrate were both enhanced (Table I).

To determine whether the synergistic effect of cystatins could be extended to other cysteine proteinase inhibitors, we also tested E64, a nonrelated-cystatin and irreversible cysteine proteinase inhibitor. E64 failed to induce any increase of nitrite production even after 48 h of incubation when added concomitantly with IFN-γ (data not shown).

Effect of Other Natural Proteinase Inhibitors on Nitrite Production—To determine whether the up-regulation of NO production by natural cysteine proteinase inhibitors was only restricted to this proteinase inhibitor family, we tested aprotinin and soybean trypsin inhibitor belonging to the serine inhibitor families, and pepstatin belonging to the aspartyl proteinase inhibitor (34) and the wide spectrum proteinase inhibitor α_2-macroglobulin (35). At the effective concentration of chicken cystatin (0.1 μM), these proteinase inhibitors did not induce NO production either on unstimulated MPM after 48 h or IFN-γ-activated MPM (data not shown), and whatever the macrophage cell lines (2C11-12N and RAW 264.7).

Complexation of the Inhibitory Site of the Cystatin with the Carboxymethylated Papain—All cystatins assayed here share the common property to inhibit cysteine proteinases. The possible influence of their proteinase inhibitory properties for NO release was studied after complete saturation of their inhibitory site by an excess of reduced-carboxymethylated papain. Inactivated papain retains the property to tightly bind the cystatin inhibitory site. The complexes of papain with chicken cystatin, human stefin B, rat T-kininogen, and D1–2 or D3 domains induced an increase of the nitrite levels identical to that of free cystatins after 48 h of incubation (Fig. 5), providing evidence that NO enhancement was unrelated to the inhibitory function of cystatins.

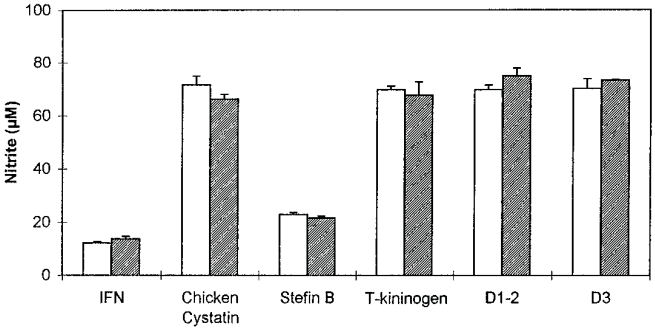

FIG. 5. **Up-regulation of the nitrite release by cystatin complexed with inactivated papain.** Papain has been inactivated by reduction and alkylation. The complete inactivation was checked by the complete loss of enzymatic activity. The carboxymethylated papain (10^{-5} M) was then mixed at 37 °C during 45 min with 10^{-6} M chicken cystatin, human stefin B, T-kininogen, and its D1–2 and D3 cystatin-like domains to generate an enzyme-inhibitor complex. The absence of cystatins in excess was controlled by the lack of inhibitory activity after addition of activated papain. Uncomplexed (*open bars*) or complexed (*filled bars*) cystatins (10^{-7} M, final concentration) were then added in the culture medium with the IFN-γ (100 IU/ml) for 48 h of incubation before measuring the nitrite levels. Means ± S.D. of one experiment performed in triplicate representative of three independent experiments.

DISCUSSION

NO produced by cytokine-activated murine macrophages during parasite infections plays a central role in the control of parasite killing (36). It has been recently shown that *T. cruzi* enhanced NO levels (9–11). The membrane-associated parasite cysteine proteinase cruzipain which is a major component involved in the *T. cruzi* host-cell infection could be involved in the process as deduced from the observation that synthetic inhibitors of cysteine proteinases decrease parasite infection (20, 21). Preliminary experiments to investigate the possible role of natural cysteine proteinase inhibitors to modulate *T. cruzi* infection and NO levels of IFN-γ-activated macrophages lead to the surprising observation that chicken cystatin was able to modulate nitrite production by IFN-γ-activated macrophages. However, cystatin-induced NO increase was observed only when MPM were activated by the IFN-γ cytokine. NO release from unstimulated MPM was not stimulated by cystatin and this remained true even after 48 h. Thus chicken cystatin cannot induce NOS synthesis from unstimulated MPM as LPS or IFN-γ and acts only synergistically with IFN-γ. The effect was clearly concentration-dependent going from a 4-fold increase in the NO production at a 10 nM concentration to a 12-fold increase at 1 μM chicken cystatin.

Other members of the cystatin superfamily, human stefin B, rat T-kininogen, and its cystatin-like domains D1–2 and D3, which belong to family 1 and 3, respectively, also demonstrated the same properties even though the concentration-dependent up-regulation of the nitrite release by 0.1 μM stefin B was about half of that with chicken cystatin. The biological effect of cystatin family members as NO synergic inducers is not related to the inhibition of a cysteine proteinase activity since the irreversible and structurally unrelated cysteine proteinase inhibitor E64, did not induce any increase in the nitrite level. Several biological compounds have been reported to act synergistically with NO inducers to induce NO production by murine macrophages. Such a synergism has been demonstrated using murine macrophages stimulated with IFN-γ and tumor necrosis factor-α or interleukin-2 (37, 38). Northern blot analysis showed that tumor necrosis factor-α or interleukin-2 dramatically increased the mRNA levels of the inducible NOS when

added with the inducers, although they are unable to induce detectable mRNA when used alone.

Some studies have shown that NO release by macrophages could be modulated by proteinase inhibitors. Kilbourn and Lorenz-Berestein (39) reported that chloromethyl ketone derivatives, which are synthetic inhibitors of serine and cysteine proteinases, decreased nitrite production by activated MPM. Similar results were found using rat alveolar macrophages (40). Soybean trypsin inhibitor, a natural serine proteinase inhibitor has been shown to induce NO release from unstimulated rat alveolar macrophages (41). At variance with these studies, all assays carried out with proteinase inhibitors other than cystatins failed to demonstrate any increase in NO release from unstimulated mouse macrophages, or any up-regulation from activated macrophages. In contrast to the studies that used synthetic inhibitors (39, 40), our experiments were performed with natural serine proteinases that are unable to cross the cell membrane and thus would be inefficient in the modulation of the enzyme activities that may regulate the NO synthesis pathway. Moreover, Chesrown *et al.* (42) studying the modulation of NOS mRNA by interleukin-10 emphasized specific cell type differences of the response forward in other studies. The fact that we used in our study MPM instead of rat alveolar macrophages (40, 41) could explain these contradictory results.

The structural reason why all cystatins may induce an increase in NO synthesis from activated MPM remains unclear. Conserved structural motifs are present within the sequence of cystatins especially in the regions that participate to the inhibitory site (43). We demonstrated here that saturation of the cystatin inhibitory site by reduced-alkylated papain did not interfere with cystatin-induced NO release from activated MPM, suggesting that the inhibitory site was not involved in the process. In addition, the equilibrium constant for dissociation of carboxymethylated papain and the chicken cystatin is in the picomolar range. NO synthase activation is induced by cystatin in the nanomolar range. It is unlikely that the complex is dissociated under the conditions of the experiments (44). Other functions have been attributed to individual cystatins, one of which is to release a biologically active peptide from their N-terminal end. This has been demonstrated for human cystatin C which releases postin, a tetrapeptide antagonist of the macrophage-activating tetrapeptide tuftsin (45). The postin sequence, however, is not found in any of the cystatins used in this work. The vasoactive peptide bradykinin which is present in T-kininogen and its D3 fragment but not in chicken cystatin and human stefin B has also been shown to be inefficient in modulating NO release from activated MPM. Whether other releasable peptides or constitutive sequences are present in cystatins which could explain this new, so far unknown property resulting in the up-regulation of NO synthesis from activated macrophages remains to be demonstrated.

Acknowledgments—We thank Dr. Duchateau and H. Collet for LPS assays.

REFERENCES

1. Lowenstein, C. J., Dinerman, J. L., and Snyder, S. H. (1994) *Ann. Intern. Med.* **120**, 227–237
2. Knowles, R. G., and Moncada, S. (1994) *Biochem. J.* **298,** 249–258
3. Nathan, C., and Xie, Q. (1994) *J. Biol. Chem.* **269,** 13725–13728
4. Stuehr, D. J., and Marletta, M. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 7738–7742
5. Stuehr, D. J., and Marletta, M. A. (1987) *J. Immunol.* **139,** 518–525
6. Lowenstein, C. J., Glatt, C. S., Bredt, D. S., and Snyder, S. H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6711–6715
7. Xie, Q. W., Cho, H. J., Calaycay, J., Munford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T., and Nathan, C. (1992) *Science* **256,** 225–228
8. Tanowitz, H. B, Kirchhoff, L. V., Simon, D., Morris, S. A., Weiss, L. M., and Wittner, M. (1992) *Clin. Microbiol. Rev.* **5,** 400–419
9. Vespa, G. N. R., Cunha, F. Q., and Silva, J. S. (1994) *Infect. Immun.* **62,** 5177–5182
10. Metz, G., Carlier, Y., and Vray, B. (1993) *Parasite Immunol.* **15,** 693–699
11. Petray, P., Rottenberg, M. E., Grinstein, S., and Orn, A. (1994) *Parasite Immunol.* **16,** 193–199
12. Araujo-Jorge, T. C. (1989) *Mem. Inst. Oswaldo Cruz.* **84,** 441–462
13. Scharfstein, J., Schechter, M., Senna, M., Peralta, J. M., Mendonça-Previato, L., and Miles, M. A. (1986) *J. Immunol.* **137,** 1336–1341
14. Cazullo, J. J., Couso, R., Raimondi, A., Wernstedt, C., and Hellman, U. (1989) *Mol. Biochem. Parasitol.* **33,** 33–42
15. Cazullo, J. J., Cazullo-Franke, M. C., Martinez, J., and Franke-de-Cazullo, B. M. (1990) *Biochem. Biophys. Acta* **1037,** 186–191
16. Murta, A. C. M., Persechini, P. M., Padron, T. D. S., de Souza, W., Guimaraes, J. A., and Scharfstein, J. (1990) *Mol. Biochem. Parasitol.* **43,** 27–38
17. Eakin, A. E., Mills, A. A., Harth, G., McKerrow, J. H., and Craik, C. S. (1992) *J. Biol. Chem.* **267,** 7411–7420
18. Souto-Padron, T., Campetella, O. E., Cazullo, J. J., and de Souza, W. (1990) *J. Cell Sci.* **96,** 485–490
19. Sakurada, J. K., Maury, A., Tamashiro, W. M. S. C., Araujo, P. M. F., Camargo, I. J. B., and Rangel, H. A. (1992) *Res. Immunol.* **143,** 157–164
20. Meirelles, M. N. L., Juliano, L., Carmona, E., Silva, S. G., Costa, E. M., Murta, A. C. M., and Scharfstein, J. (1992) *Mol. Biochem. Parasitol.* **52,** 175–184
21. Harth, G., Andrew, N., Mills, A. A., Engel, J. C., Smith, R., and McKerrow, J. H. (1993) *Mol. Biochem. Parasitol.* **58,** 17–24
22. Scharfstein, J., Abrahamson, M., Palatnik de Souza, C. B., Barral, A., and Silva, I. V. (1995) *J. Immunol. Methods* **182**, 63–72
23. Bode, W., Engh, R., Musil, D., Thiele, U., Huber, R., Karshikov, A., Brzin, J., Kos, J., and Turk, V. (1988) *EMBO J.* **7,** 2593–2599
24. Machleidt, W., Thiele, U., Laber, B., Assfalg-Machleidt, I., Esterl, A., Wiegand, G., Kos, J., Turk, V., and Bode, W. (1989) *FEBS Lett.* **243,** 234–238
25. Engh, R. A., Dieckmann, T., Bode, W., Auerswald, E. A., Turk, V., Huber, R., and Oschkinat, H. (1993) *J. Mol. Biol.* **234,** 1060–1069
26. Moreau, T., Gutman, N., Faucher, D., and Gauthier, F. (1989) *J. Biol. Chem.* **264**, 4298–4303
27. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982) *Anal. Biochem.* **126,** 131–138
28. Deleted in proof
29. Denis, M. (1994) *J. Leukocyte Biol.* **55,** 682–684
30. Turk, V., and Bode, W. (1991) *FEBS Lett.* **285,** 213–219
31. Gutman, N., Moreau, T., Alhenc-Gelas, F., Baussant, T., El Moujahed, A., Akpona, S., and Gauthier, F. (1988) *Eur. J. Biochem.* **171,** 577–582
32. Tiffany, C. W., and Burch, R. M. (1989) *FEBS Lett.* **247,** 189–192
33. Bogle, R. G., Coade, S. B., Moncada, S., Pearson, J. D., and Mann, G. E. (1991) *Biochem. Biophys. Res. Commun.* **180,** 926–932
34. Bode, W., and Huber, R. (1992) *J. Biochem.* **204,** 433–451
35. Borth, W. (1992) *FASEB J.* **6,** 3345–3353
36. Oswald, I. P., Wynn, T. A., Sher, A., and James, S. L. (1994) *Comp. Biochem. Physiol.* **108C,** 11–18
37. Weisz, A., Ogushi, S., Cicatiello, L., and Esumi, H. (1994) *J. Biol. Chem.* **269,** 8324–8433
38. Deng, W., Thiel, B., Tannenbaum, C. S., Hamilton, T. A., and Stuehr, D. J. (1993) *J. Immunol.* **151**, 322–329
39. Kilbourn, R., and Lopez-Berestein, G. (1990) *J. Immunol.* **144,** 1042–1045
40. Jorens, P. G., Van Overveld, F. J., Bult, H., Vermeire, P. A., and Herman, A. G. (1992) *Agents Actions* **36,** 243–247
41. Jorens, P. G., Van Overveld, F. J., Bult, H., Vermeire, P. A., and Herman, A. G. (1992) *Biochem. Pharmacol.* **44,** 387–390
42. Chesrown, S. E., Monnier, J., Visner, G., and Nick, H. S. (1994) *Biochem. Biophys. Res. Commun.* **200,** 126–134
43. Barrett, A. J., Rawlings, N. D., Davies, M. E., Machleidt, W., Salvesen, G., and Turk, V. (1986) in *Proteinase Inhibitors* (Barrett, A. J., and Salvesen, G., eds) pp. 515–569, Elsevier, Amsterdam
44. Björk, I., and Ylinenjärvi, K. (1989) *Biochem. J.* **260,** 61–68
45. Leung-Tack, J., Tavera, C., Gensac, M. C., Martinez, J., and Collé, A. (1990) *Exp. Cell Res.* **188,** 16–22