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Chromogranin A protects vessels against tumor necrosis factor α-induced vascular leakage

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ABSTRACT

Elevated levels of circulating chromogranin A (CgA), a protein stored in the secretory granules of many neuroendocrine cells and neurons, have been detected in the blood of patients with neuroendocrine tumors or heart failure. The pathophysiological role of increased secretion of CgA is unknown. Using mice bearing subcutaneous tumors genetically engineered to secrete CgA in circulation, we have found that increased blood levels of this protein prevent vascular leakage induced by tumor necrosis factor-α (TNF) in the liver venous system. Structure–activity studies, carried out with CgA fragments administered to normal mice, showed that an active site is located within residues 7–57 of CgA. Accordingly, an anti-CgA antibody directed to residues 53–57 inhibited the effect of circulating CgA, either endogenously produced or exogenously administered, on liver vessels. Studies of the mechanism of action showed that CgA inhibits TNF-induced VE-cadherin down-regulation and barrier alteration of cultured endothelial cells, in an indirect manner. Other effectors, such as thrombin and vascular endothelial growth factor were partially inhibited by CgA N-terminal fragments in in vitro permeability assays. These findings suggest that circulating CgA could help regulate the endothelial barrier function and to protect vessels against TNF-induced plasma leakage in pathological conditions characterized by increased production of TNF and CgA, such as cancer or heart failure.

Key words: Chromogranin A • tumor necrosis factor • endothelial cell • vascular permeability • heart failure

Chromogranin A (CgA) is an acidic glycoprotein belonging to a family of regulated secretory proteins stored in the dense core granules of the adrenal medulla and of many other neuroendocrine cells and neurons (1, 2). This protein is believed to play a pivotal role in secretory granule biogenesis and hormone packaging (3). After an appropriate stimulus, CgA is released into the extracellular environment, together with coresident hormones, and reaches the bloodstream via capillaries or lymphatic vessels (1). Elevated levels of circulating CgA have been detected in the blood of patients with endocrine and neuroendocrine tumors (4–6) and renal failure (7). Moreover, we have recently shown that circulating CgA is markedly

increased in patients with heart failure, depending on the severity of the diseases, and that it correlates with tumor necrosis factor-α (TNF) and TNF soluble receptors (8, 9). It has been proposed that this protein is a precursor of biologically active peptides with endocrine, paracrine, and autocrine functions (6). For instance, N-terminal fragments corresponding to amino acids 1–76 and 1–113 are released by adrenal medulla and sympathetic nerve terminals (10) and can suppress vasoconstriction in isolated blood vessels (10, 11). Moreover, a fragment corresponding to residues 1–78 can induce adhesion and spreading of fibroblasts and smooth muscle cells on solid phases, suggesting a role for CgA in modulating cell adhesion (12–14). In a preliminary work, we also observed that chromogranin A can affect the TNF-induced changes of cell shape and barrier function of endothelial cell monolayers (15).

These findings, together with the notion that the endothelial lining of vessels is exposed to circulating CgA secreted by normal or neoplastic neuroendocrine cells or to CgA released by sympathetic nerve terminals, prompted us to investigate whether endogenously produced CgA could affect vascular leakage. We show here that increased levels of endogenous CgA can inhibit, at a systemic level, the vascular leakage induced by TNF, a cytokine implicated in a variety of inflammatory diseases, including heart failure (16–20) and cancer (21). We propose that pathophysiological secretion of this protein could contribute to regulate TNF-induced vascular leakage in pathological conditions characterized by increased production of both TNF and CgA, as it occurs in patients with (neuro)endocrine tumors or heart failure.

MATERIALS AND METHODS

Cell cultures and reagents

Human Umbilical Vein Endothelial cells (HUVEC) were isolated from human umbilical veins by collagenase treatment as described (22) and cultured in 1% gelatin-coated flasks (Falcon, Becton Dickinson, Bedford, MA) containing endotoxin-free Medium 199 (Sigma, St. Louis, MO), 20% heat-inactivated fetal calf serum (FCS, Hyclone, Logan, UT), 1% bovine retina derived growth factor, 90 μg/ml heparin, 100 IU/ml penicillin, 100 μg/ml streptomycin (Biochrom, Berlin, Germany) (complete medium, CM). All experiments were carried out with HUVEC at passage 2–4. Mouse anti-CgA mAb 5A8 (IgG1) was described previously (14). Human vascular endothelial growth factor, isoform 165 (VEGF), was from PeproTech (Rocky Hill, NJ). Thrombin and horseradish peroxidase (HRP) were from Sigma. Human TNF, ($5.45\pm3.1\times10^7$) units/mg, and murine TNF, ($1.2\pm0.14\times10^8$) units/mg, were prepared by recombinant DNA techniques as described (23). CgA-secreting RMA-3A3 and nonsecreting RMA-WT mouse lymphoma cells were generated by genetic engineering technology as described previously (24). The production of CgA in culture supernatant was checked by ELISA as described (24).

Production of natural CgA and recombinant N-terminal fragments

Natural human CgA was purified from pheochromocytoma tissue extracts (heat stable fraction) by immunoaffinity chromatography, essentially as described previously (25). SDS-PAGE of the final product revealed two bands of 70 and 60 kDa. Recombinant CgA1-78 fragment was obtained by expression in *E. coli* (26) and purified by reverse-phase HPLC and gel filtration chromatography as described previously (14). SDS-PAGE of CgA1-78 showed that this product

was homogeneous under reducing and nonreducing conditions. The molecular mass of CgA1-78, as measured by electrospray mass spectrometry, was 9069.7 Da (expected 9069.3 Da). Endotoxin content was 0.008–0.016 units/μg by the Limulus Amoebocyte Lysate (LAL) Pyrotest (Difco Laboratories, Detroit, MI). Synthetic peptide CgA7-57 was prepared as described (14).

In vivo permeability assay

Animal studies were carried out in accordance with institutional animal care guidelines. The effect of murine TNF on the leakage of trypan blue-albumin complex from liver vessels was evaluated in CD-1 nu/nu BR (nude) mice (Charles River Laboratories, Calco, Italy) bearing CgA-secreting and CgA nonsecreting tumors. To this aim, female mice were challenged with 2×10^6 RMA-3A6 (CgA-positive) or RMA-WT cells (CgA-negative), s.c. in the left flank. After 15 days, tumor-bearing mice were used for the in vivo permeability assay (27), as follows: murine TNF in 0.2 ml of 0.9% sodium chloride, was administered intraperitoneally to anesthetized mice. After 30 min, the liver of each animal was perfused for 5 min with 4 ml of a solution containing 0.4% bovine serum albumin, 0.5% trypan blue, 0.85% sodium chloride through the inferior vena cava. The perfusate was drained from the portal vein, as described (28). Then, 5 ml of 0.9% sodium chloride were injected through the inferior vena cava, to remove the dye present in the lumen of vessels. The livers were dissected, homogenized with 3 ml of PBS, and centrifuged. The absorbance at 540 nm of supernatants was then measured. The effect of TNF on vascular leakage was also evaluated in normal male BALB/c mice (Charles River Italia, Calco, Italy) using murine TNF alone or in combination with other agents such as CgA, CgA fragments, or anti-CgA antibodies (see "Results").

TNF dose–response experiments showed that in these models vascular leakage can be induced 30 min after administration of 2 ng of TNF. Little or no soluble p55– or p75-TNF receptor shedding was observed in circulation 1 h after administration of TNF, as checked by ELISA (data not shown). Because administration of higher doses (10–100 ng) was accompanied by the release of soluble p75 TNF receptor in circulation, the dose of 2 ng was chosen for all experiments.

TNF cytotoxicity assay

The cytotoxic activity of TNF in the presence or absence of CgA was estimated by standard cytolytic assays using L-M mouse fibroblasts (ATCC CCL1.2) as described (29).

Confocal microscopy

Cytoskeletal studies were performed using HUVEC (1×10^5 cells in CM grown on glass coverslips for five days) incubated for 2 h with TNF alone, or in combination with CgA1-78 or CgA, in CM. The cells were fixed with 2% paraformaldehyde for 10 min, permeabilized with 0.01% Triton X-100 in PBS and incubated with FITC-phalloidin (Sigma) to stain F-actin. Staining of VE-cadherin was obtained with mAb BV9 (kindly provided by Monica Corada, IFOM, Milan, Italy) followed by FITC-anti-mouse immunoglobulins (Dako, Denmark). To assess CgA internalization, HUVEC were treated with FITC-labeled CgA1-78 for 4 h at 37°C, fixed and, when indicated, counterstained with anti-CD31 mAb M89D3, followed by TRITC-rabbit anti-mouse immunoglobulins (Dako). Coverslips were mounted using 50% glycerol in

PBS. Microscopic analysis was performed using a Bio-Rad MRC 1000 confocal scanning microscope (Biorad Laboratories, Milan, Italy) and × 63 objective. Fluorescence digital images were recorded.

In vitro permeability assay

The assay was carried out by measuring the flux of radiolabeled albumin through HUVEC monolayers, cultured on gelatin-coated membranes of Transwell cell culture chambers (0.4 μm filters, Costar, Cambridge, MA). HUVEC (5×10^4 cells/well, in CM) were cultured for 4 days in the upper compartment of the Transwell device. The culture medium was then replaced with Medium 199 containing 1% FCS. One day later, confluent cells were treated for 2 h with 4 ng/ml human TNF solution, either in the absence or in the presence of CgA or N-terminal fragments, in Medium 199 containing 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (200 μl/well). Alternatively, the cells were treated for 20 min with 2 U/ml thrombin or 20 ng/ml VEGF in the same medium. HUVEC were then incubated for 10 min with 1.5 mg/ml human serum albumin (Farma-Biagini SpA, Lucca, Italy) to saturate albumin receptors and then washed. After washing, 4 μCi/ml ^{125}I-labeled bovine serum albumin (NEN, Boston, MA) was added to the upper compartment (50 μl/well). The lower compartment was then filled with Medium 199 containing 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (600μl/well) and incubated for 1 h with gentle orbital agitation. At various times, samples (30 μl) were taken from the lower compartment and their radioactivity was measured using a γ-counter (Packard, Sterling, VA).

RESULTS

Increased production of endogenous CgA protect vessels against TNF-induced plasma leakage in the liver

To investigate whether pathological levels of endogenous CgA could affect TNF-induced vascular leakage we used an animal model based on mice bearing subcutaneous RMA-3A6 mouse lymphoma, which we genetically engineered to express and secrete human CgA (24). This model was characterized previously and shown to release 0.5–1.5 μg/ml (7–20 nM) human CgA in the blood 15 days after implantation (24). These levels are similar to those found in patients with neuroendocrine tumors (4–6) or with severe heart failure (8, 9). In parallel, we used mice bearing the RMA-WT (wild-type) nonsecreting tumors. To assess the effect of TNF and CgA on vascular leakage, these animals were treated with murine TNF (i.p.) and the leakage of trypan blue-albumin from liver vessels was measured 30 min later. Intraperitoneal administration of TNF induced an increase of trypan blue-albumin leakage in the liver of mice bearing CgA-negative RMA-WT tumors, but not in mice bearing CgA-positive RMA-3A6 tumors (Fig. 1*a*). Noteworthy, coadministration of TNF with an anti-CgA antibody (mAb 5A8), but not with a control antibody (mAb 9E10), induced vascular leakage also in RMA-3A6 bearing mice (Fig. 1*a*), indicating that the effect was mediated by CgA and not by other tumor derived products. Since the tumors were implanted subcutaneously, that is, at sites distant from the liver, these results suggest that the release of increased amounts of CgA in circulation can regulate the effect of TNF on vascular leakage at a systemic level. The lack of vessel protection in animals bearing CgA-nonsecreting tumors suggests that normal levels of circulating murine CgA were not sufficient to inhibit TNF.

Normal levels of endogenous CgA are not sufficient to protect vessels against TNF-induced plasma leakage

To confirm that normal levels of CgA cannot significantly inhibit TNF-induced vascular leakage, at least at a systemic level, further experiments were performed using normal mice. To this aim we measured the extravasation of trypan blue-albumin in the liver of normal mice after treatment with TNF, alone or in combination with the neutralizing anti-CgA mAb 5A8, taking advantage from the fact that this antibody cross-reacts with murine CgA (24). Neither mAb 5A8 nor a control antibody (mAb 9E10) was able to neutralize the TNF-induced leakage of dye (Fig. 1*b*). Since 5A8 was able to inhibit the effect of pathological levels of CgA, these and the above results suggest that normal levels of circulating CgA are not sufficient to affect the activity of TNF at a systemic level.

The bioactive site of CgA is located within residues 7–57

CgA is a prohormone precursor that can be converted into various proteolytic fragments by intracellular and extracellular proteases. We have shown previously that the region 7-57 contains a site able to regulate cell adhesion and shape, at least in the case of fibroblasts and smooth muscle cells. To assess whether the region 7-57 contains also the site that protects vessels from vascular leakage, we compared the effect of natural human CgA, recombinant CgA1-78, and synthetic CgA7-57 (exogenously administered) on the vascular leakage induced by TNF in normal mice (Table 1 and Fig. 2). As expected, TNF-induced marked dye entry into liver parenchyma, whereas CgA or CgA fragments, alone, did not affect dye extravasation (Fig. 2). Preadministration of CgA (0.7 μg), CgA1-78 or CgA7-57 (3 μg), significantly inhibited TNF-induced dye extravasation (Table 1). Coadministration of the anti-CgA mAb 5A8 partially neutralized the effect of CgA and CgA1-78, whereas control antibodies (mAb 19E12 or mAb 9E10) were inactive. Because mAb 5A8 was shown previously to bind an epitope located within residues 53-57 of CgA, these results, together with the inhibitory activity of CgA7-57, suggest that the active site of circulating CgA is located within residues 7-57.

Mechanism of action

The mechanism of action was then investigated. In previous work, we showed that the interaction of TNF with the p55-TNF-receptor is necessary and sufficient for triggering an increase in HUVEC monolayer permeability and that the p75-TNF receptor may contribute by enhancing the response in vivo (30). Thus, we investigated whether CgA could bind TNF or TNF receptors and, consequently, inhibit the TNF/TNF-receptor interactions. We observed no binding of human TNF to CgA or CgA1-78, immobilized onto microtiter plates, by ELISA with anti-TNF polyclonal IgGs (not shown). Moreover, neither CgA nor CgA1-78 inhibited the cytolytic activity of human TNF (a selective murine p55-TNF-receptor agonist) or murine TNF (a p55- and p75-TNF-receptor agonist) against mouse L-M cells in a standard cytotoxicity assay. Because we showed previously that the cytolytic activity of murine TNF on these cells is dependent on both the p55 and p75 receptors (31), these results imply that CgA is unable to block the binding of TNF to both membrane receptors.

It is believed that the effect of TNF on vascular leakage is primarily related to alteration of the endothelial barrier. For instance, TNF is known to promote changes in the size and shape of

endothelial cells by converting peripheral actin bundles into stress fibers (32). Moreover, TNF can induce changes in VE–cadherin distribution at intercellular adherence junctions (33) and alter endothelial permeability (32). Thus, to assess whether the effect of CgA on TNF-induced vascular leakage was related to regulation of endothelial function, we investigated the effect of CgA1-78 and CgA on TNF-induced *1*) cytoskeleton rearrangement, *2*) VE-cadherin down-modulation and *3*) permeability increase, using in vitro cultured HUVEC monolayers.

Confocal microscopy studies showed that human TNF, as expected, induces marked actin reorganization (Fig. 3*a* and *b*). Three μg/ml CgA1-78 (330 nM) inhibited actin reorganization, peripheral bundles being observed both in the absence and presence of TNF (Fig. 3*c* and *d*). The inhibition was reverted by the anti-CgA mAb 5A8, as indicated by stress fibers formation in the presence of a mixture of this antibody, CgA1-78 and TNF (Fig. 3*e*). Similarly, 3 μg/ml CgA (43 nM) inhibited TNF-induced stress fiber formation (not shown). TNF decreased VE-cadherin staining along cellular junctions (Fig. 4*a* and *b*), whereas no changes were observed with TNF in combination with CgA1-78 (Fig. 4*c* and *d*). Also, in this case, the inhibitory effect of CgA1-78 was reverted by mAb 5A8 (Fig. 4*e* and *f*).

The effect of TNF and CgA on endothelial permeability was studied by measuring their activity on the flux of radio-labeled albumin through confluent HUVEC monolayers, using the double chamber permeability assay. In this experimental model, brief incubation of cells with TNF (4 ng/ml, 30 min) was not sufficient to increase HUVEC permeability in vitro (data not shown). However, when we prolonged the incubation to 2 h, we observed a modest, but significant, increase of permeability (Fig. 5*a*). This effect was significantly inhibited by 3 μg/ml CgA1-78 (Fig. 5*a*) and by CgA in a dose-dependent manner, at concentrations consistent with pathological levels of endogenous CgA (Fig. 5*d*). Noteworthy, neither recombinant CgA1-78 nor natural CgA alone could affect the transendothelial flux of ^{125}I-albumin (Fig. 5*b* and *c*). Moreover, a mixture of 3 μg/ml CgA (43 nM) and 30 μg/ml anti-CgA 5A8 (200 nM) behaved like 0.3 μg/ml CgA (Fig. 5*e*), indicating that this antibody could block most of the CgA activity.

In conclusion, these results suggest that CgA and its N-terminal fragment can interact with endothelial cells and inhibit TNF-induced changes in intercellular adhesion molecules and permeability.

The interaction of CgA1-78 with endothelial cells was further investigated by confocal microscopy. To this purpose HUVEC monolayers were incubated with various FITC-labeled compounds for 4 h at 37°C before analysis. No cell staining occurred with FITC-streptavidin (Fig. 6*b*), whereas a dotlike signal (green) was observed with FITC-CgA1-78 (Fig. 6*a*). Counter-staining of CD31, an endothelial cell surface marker, with an anti-CD31 mAb (red) suggests that the localization of FITC-CgA1-78 was intracellular (Fig. 6*a*). This hypothesis was further supported by the observation that FITC-CgA1-78 and Alexa 568-transferrin conjugate (red), a marker of endocytotic vesicles, partially colocalized when both products were added to the cells (Fig. 6*c*–*g*). These results indicate that CgA1-78 is internalized by endothelial cells.

CgA1-78 inhibits thrombin- and VEGF-induced permeability of HUVEC monolayers

To assess whether the effect of CgA on endothelial cells was restricted to TNF activity, we investigated the effect of its N-terminal fragments on thrombin- and VEGF-induced permeability

of HUVEC monolayers. We found that the activity of thrombin was partially, but significantly, inhibited by recombinant CgA1-78 (Fig. 7*a*). Similarly, the effect of VEGF was inhibited by recombinant CgA1-78, as well by synthetic CgA7-57 (Fig. 7*b* and *c*). However, when we studied the effect of VEGF in vivo, using the liver vascular leakage model, we observed no inhibition (data not shown).

DISCUSSION

The results of this study show that circulating CgA can inhibit TNF-induced leakage of solutes from liver vessels in mouse models when it is overproduced. These findings suggest that CgA contributes to the regulation of vessel barrier function in pathological processes characterized by increased secretion of TNF and CgA, such as in patients with heart failure or (neuro)endocrine tumors. Given that CgA is concentrated and stored within secretory granules and rapidly released by neuroendocrine cells and neurons after an appropriate neural or hormonal stimulus, this protein could be an important part of neuroendocrine mechanisms that control the proinflammatory activity of TNF.

CgA is present in neuroendocrine secretory vesicles at very high concentrations, approaching millimolar levels (34). Thus, after secretion, CgA could affect endothelial cells by paracrine mechanisms, considering the relatively high concentration in the secretory cell microenvironment. CgA is also present in the blood of normal subjects at 0.5–2 nM (8, 35) and up to 15–30 nM in patients with severe heart failure (8, 9) or neuroendocrine tumors (36, 37). Because we observed in our in vitro and in vivo assays TNF inhibition at concentrations ranging from 4–40 nM, it is possible that pathological concentrations of circulating CgA are functionally active at a systemic level. This hypothesis is supported by the results obtained with mice bearing subcutaneous mouse lymphomas, which, we genetically engineered to express and secrete human CgA in the blood stream. In these models, we observed that TNF-induced leakage of macromolecules in the liver is inhibited in mice bearing CgA-secreting tumors, but not in nonsecreting tumor-bearing mice. Moreover, a neutralizing anti-CgA antibody increased the TNF-induced leakage in mice bearing CgA-secreting tumors, but not in normal mice. These results point to systemic effects of CgA only in conditions of increased production, as it may occur in patients with neuroendocrine tumors or heart failure. Interestingly, we recently found that the increase of circulating CgA in patients with heart failure correlates with the release in circulation of TNF and soluble TNF receptors and that all these molecules are important prognostic markers (8, 9, 38). Although the clinical significance of TNF production in heart failure patients remains uncertain, its ability to induce cachexia, left ventricular dysfunction and pulmonary edema, suggests that TNF, in concert with other inflammatory cytokines, could play a pathogenetic role (16–20). It is possible that the regulated secretion of CgA, in concert with TNF soluble receptors, contribute to reduce the potentially dangerous effects of pathological levels of TNF.

The results of structure-function studies, carried out with natural, recombinant, and synthetic fragments indicate that the N-terminal region of CgA7-57 contains the active site. In previous work we showed that CgA7-57 can induce fibroblast adhesion to solid phases (14). In particular, the region 47-57 was found to contain a site critical for fibroblast adhesion. The importance of the region 47-57 for the biological activity of CgA is also reflected by the high degree of homology between human, rat, mouse, porcine, ostrich, and frog CgA, compared with other

regions (39–41). Interestingly, mAb 5A8, a monoclonal antibody directed to residues Arg53, His54, and Leu57, blocks the activity of CgA on vascular leakage as well as the proadhesive activity of CgA N-terminal fragments on fibroblasts (14). Other studies showed that CgA N-terminal fragments inhibits the miogenic tone in isolated conduit vessels and resistance arteries (11, 42, 43) and exert negative inotropic effects (44). However, whether these effects are mediated by the same site or not remains to be investigated.

We have also investigated the mechanism of action and the cellular targets of CgA/TNF in vascular leakage. It is known that TNF can induce vascular leakage by interacting with the p55 TNF-receptor on endothelial cells (30). Thus, it is possible that the endothelial lining of vessels is the primary site of action also of CgA. Using cultured HUVEC monolayers we observed that CgA and its N-terminal fragments can inhibit TNF-induced cytoskeletal reorganization, intercellular VE-cadherin down-modulation (a transmembrane protein important for cell–cell adhesion) and barrier alteration, suggesting that the endothelial lining of vessels can indeed be affected by CgA.

Concerning the molecular mechanism of TNF inhibition, the results of our study suggest that CgA does not act by blocking the TNF/TNF-receptor interactions. More likely, CgA interacts with other components of endothelial cells. It has been reported that bovine aorta endothelial cells bind and internalize 1 nM ^{125}I-labeled CgA; both the binding and internalization are temperature- and time-dependent, reaching a maximum after 2 h of incubation (45). Although a specific receptor was not identified, this study showed that endothelial cells can bind CgA with low affinity and high capacity. The results of confocal microscopy studies showed that HUVEC internalize CgA1-78. Although internalization is not necessarily linked to changes in permeability, this result supports the concept that CgA can indeed interact with endothelial cells.

The finding that CgA1-78 could also partially inhibit thrombin-induced and VEGF-induced permeability of HUVEC may have several explanations: For instance, CgA could act on signaling components of endothelial cells that are common to TNF and these effectors. Alternatively, these phenomena are the result of enhanced surface expression or function of adhesion molecules, for example, VE-cadherin, in a manner that cannot be overcome by TNF, thrombin, or VEGF signaling. The physiological relevance of this finding, however, remains to be established, as we failed to demonstrate inhibition of VEGF permeability in our in vivo model. It is likely that other factors are critical for the differential regulation of TNF, VEGF, thrombin, and CgA activity in vivo. Similarly, when we studied the effect of CgA1-78 on carrageenan-induced hindpaw edema in rats we did not observe significant inhibition (data not shown), pointing to a specific mechanism for TNF.

In conclusion, we have found that increased levels of circulating CgA protect vessels from TNF-induced vascular leakage in animal models. We propose that increased (neuro)endocrine secretion of CgA could contribute to protect vessels against TNF-induced plasma leakage in pathological conditions characterized by increased production of TNF and CgA, such as in patients with severe heart failure and cancer. The results of this work could stimulate further studies aimed at assessing whether exogenously administered CgA can reduce the vascular leakage in inflammatory diseases associated with overproduction of TNF.

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REFERENCES

1. Winkler, H., and Fischer-Colbrie, R. (1992) The chromogranins A and B: The first 25 years and future perspectives. *Neuroscience* **49,** 497–528

2. Rosa, P., and Gerdes, H. H. (1994) The granin protein family: markers for neuroendocrine cells and tools for the diagnosis of neuroendocrine tumors. *J. Endocr. Invest.* **17,** 207–225

3. Kim, T., Tao-Cheng, J. H., Eiden, L. E., and Loh, Y. P. (2001) Chromogranin A, an "on/off"; Switch controlling dense-core secretory granule biogenesis. *Cell* **106,** 499–509.

4. O'Connor, D. T., and Bernstein, K. N. (1984) Radioimmunoassay of chromogranin A in plasma as a measure of exocytotic sympathoadrenal activity in normal subjects and patients with pheochromocytoma. *New Engl. J. Med.* **311,** 764–770

5. Sobol, R. E., Memoli, V., and Deftos, L. J. (1989) Hormone-negative, chromogranin A-positive endocrine tumors. *New Engl. J. Med.* **320,** 444–447

6. Deftos, L. J. (1991) Chromogranin A: Its role in endocrine function and as an endocrine and neuroendocrine tumor marker. *Endocr. Rev.* **12,** 181–187

7. Ziegler, M. G., Kennedy, B., Morrissey, E., and O'Connor, D. T. (1990) Norepinephrine clearance, chromogranin A and dopamine beta hydroxylase in renal failure. *Kidney Int.* **37,** 1357–1362

8. Corti, A., Ferrari, R., and Ceconi, C. (2000) Chromogranin A and tumor necrosis factor alpha in heart failure. *Adv. Exp. Med. Biol.* **482,** 351–359

9. Ceconi, C., Ferrari, R., Bachetti, T., Opasich, C., Volterrani, M., Colombo, B., Parrinello, G., and Corti, A. (2002) Chromogranin A in heart failure; A novel neurohumoral factor and a predictor for mortality. *Eur. Heart J.* **23,** 967–974.

10. Helle, K. B., Marley, P. D., Angeletti, R. H., Aunis, D., Galindo, E., Small, D. H., and Livett, B. G. (1993) Chromogranin A: Secretion of processed products from the stimulated retrogradely perfused bovine adrenal gland. *J. Neuroendocr.* **5,** 413–420

11. Aardal, S., Helle, K. B., Elsayed, S., Reed, R. K., and Serck-Hanssen, G. (1993) Vasostatins, comprising the N-terminal domain of chromogranin A, suppress tension in isolated human blood vessel segments. *J. Neuroendocr.* **5,** 405–412

12. Gasparri, A., Sidoli, A., Sanchez, L. P., Longhi, R., Siccardi, A. G., Marchisio, P. C., and Corti, A. (1997) Chromogranin A fragments modulate cell adhesion. Identification and characterization of a pro-adhesive domain. *J. Biol. Chem.* **272,** 20,835–20,843

13. Colombo, B., Longhi, R., Marinzi, C., Magni, F., Cattaneo, A., Yoo, S. H., Curnis, F., and Corti, A. (2002) Cleavage of chromogranin A N-terminal domain by plasmin provides a new mechanism for regulating cell adhesion. *J. Biol. Chem.* **277,** 45,911–45,919.

14. Ratti, S., Curnis, F., Longhi, R., Colombo, B., Gasparri, A., Magni, F., Manera, E., Metz-Boutigue, M. H., and Corti, A. (2000) Structure-activity relationships of chromogranin A in cell adhesion. Identification and characterization of an adhesion site for fibroblasts and smooth muscle cells. *J. Biol. Chem.* 275, 29,257–29,263

15. Ferrero, E., Magni, E., Curnis, F., Villa, A., Ferrero, M. E., and Corti, A. (2002) Regulation of endothelial cell shape and barrier function by chromogranin A. *Ann. N.Y. Acad. Sci.* **971,** 355–358.

16. Hegewisch, S., Weh, H. J., and Hossfeld, D. K. (1990) TNF-induced cardiomyopathy. *Lancet* **335,** 294–295

17. Mann, D. L. (1996) The effect of tumor necrosis factor-alpha on cardiac structure and function: a tale of two cytokines. *J. Cardiac Fail.* **2,** S165–172

18. Lo, S. K., Everitt, J., Gu, J., and Malik, A. B. (1992) Tumor necrosis factor mediates experimental pulmonary edema by ICAM-1 and CD18-dependent mechanisms. *J. Clin. Invest.* **89,** 981–988

19. Yokoyama, T., Vaca, L., Rossen, R. D., Durante, W., Hazarika, P., and Mann, D. L. (1993) Cellular basis for the negative inotropic effects of tumor necrosis factor-alpha in the adult mammalian heart. *J. Clin. Invest.* **92,** 2303–2312

20. Eichenholz, P. W., Eichacker, P. Q., Hoffman, W. D., Banks, S. M., Parrillo, J. E., Danner, R. L., and Natanson, C. (1992) Tumor necrosis factor challenges in canines: patterns of cardiovascular dysfunction. *Am. J. Physiol.* **263,** H668–675

21. Balkwill, F., Osborne, R., Burke, F., Naylor, S., Talbot, D., Durbin, H., Tavernier, J., and Fiers, W. (1987) Evidence for tumour necrosis factor/cachectin production in cancer. *Lancet* **2,** 1229–1232.

22. Ferrero, E., Ferrero, M. E., Pardi, R., and Zocchi, M. R. (1995) The platelet endothelial cell adhesion molecule-1 (PECAM1) contributes to endothelial barrier function. *FEBS Lett.* **374,** 323–326

23. Curnis, F., Sacchi, A., Borgna, L., Magni, F., Gasparri, A., and Corti, A. (2000) Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). *Nat. Biotechnol.* **18,** 1185–1190

24. Colombo, B., Curnis, F., Foglieni, C., Monno, A., Arrigoni, G., and Corti, A. (2002) Chromogranin a expression in neoplastic cells affects tumor growth and morphogenesis in mouse models. *Cancer Res.* **62,** 941–946.

25. Corti, A., Longhi, R., Gasparri, A., Chen, F., Pelagi, M., and Siccardi, A. G. (1996) Antigenic regions of human chromogranin A and their topographic relationships with structural/functional domains. *Eur. J. Biochem.* **235,** 275–280

26. Corti, A., Sanchez, L. P., Gasparri, A., Flavio, C., Longhi, R., Brandazza, A., Siccardi, A., and Sidoli, A. (1997) Production and structure characterization of recombinant chromogranin A N-terminal fragments (vasostatins): evidence of dimer-monomer equilibria. *Eur. J. Biochem.* **248,** 692–699

27. Ferrero, E., Villa, A., Ferrero, M. E., Toninelli, E., Bender, J. R., Pardi, R., and Zocchi, M. R. (1996) Tumor necrosis factor alpha-induced vascular leakage involves PECAM1 phosphorylation. *Cancer Res.* **56,** 3211–3215

28. Branster, M. V., and Morton, R. K. (1957) Isolation of intact liver cells. *Nature (London)* **180,** 1283–1284

29. Corti, A., Poiesi, C., Merli, S., and Cassani, G. (1994) Tumor necrosis factor (TNF) alpha quantification by ELISA and bioassay: effects of TNF alpha-soluble TNF receptor (p55) complex dissociation during assay incubations. *J. Immunol. Meth.* **177,** 191–198

30. Ferrero, E., Zocchi, M. R., Magni, E., Panzeri, M. C., Curnis, F., Rugarli, C., Ferrero, M. E., and Corti, A. (2001) Roles of tumor necrosis factor p55 and p75 receptors in TNF-alpha-induced vascular permeability. *Am. J. Physiol. Cell Physiol.* **281,** C1173–1179.

31. Pelagi, M., Curnis, F., Colombo, B., Rovere, P., Sacchi, A., Manfredi, A. A., and Corti, A. (2000) Caspase inhibition reveals functional cooperation between p55- and p75-TNF receptors in cell necrosis. *Eur. Cytokine Net.* **11,** 580–588

32. Goldblum, S. E., Ding, X., and Campbell-Washington, J. (1993) TNF-alpha induces endothelial cell F-actin depolymerization, new actin synthesis, and barrier dysfunction. *Am. J. Physiol.* **264,** C894–905

33. Friedl, J., Puhlmann, M., Bartlett, D. L., Libutti, S. K., Turner, E. N., Gnant, M. F., and Alexander, H. R. (2002) Induction of permeability across endothelial cell monolayers by tumor necrosis factor (TNF) occurs via a tissue factor-dependent mechanism: relationship between the procoagulant and permeability effects of TNF. *Blood* **100,** 1334–1339.

34. Winkler, H., & Westhead, E., (1980) The molecular organization of adrenal chromaffin granules. *Neuroscience* **5,** 1803–1823.

35. Wu, T. L., Chang, C. P., Tsao, K. C., Sun, C. F., and Wu, J. T. (1999) Development of a microplate assay for serum chromogranin A (CgA): establishment of normal reference values and detection of elevated CgA in malignant diseases. *J. Clin. Lab. Anal.* **13,** 312–319

36. Stridsberg, M., Oberg, K., Li, Q., Engstrom, U., and Lundqvist, G. (1995) Measurements of chromogranin A, chromogranin B (secretogranin I), chromogranin C (secretogranin II) and pancreastatin in plasma and urine from patients with carcinoid tumours and endocrine pancreatic tumours. *J. Endocr.* **144,** 49–59

37. Nobels, F. R., Kwekkeboom, D. J., Coopmans, W., Schoenmakers, C. H., Lindemans, J., De Herder, W. W., Krenning, E. P., Bouillon, R., and Lamberts, S. W. (1997) Chromogranin A as serum marker for neuroendocrine neoplasia: comparison with neuron-specific enolase and the alpha-subunit of glycoprotein hormones. *J. Clin. Endocr. Metab.* **82,** 2622–2628

38. Ferrari, R., Bachetti, T., Confortini, R., Opasich, C., Febo, O., Corti, A., Cassani, G., and Visioli, O. (1995) Tumor necrosis factor soluble receptors in patients with various degrees of congestive heart failure. *Circulation* **92,** 1479–1486

39. Simon, J. P., and Aunis, D. (1989) Biochemistry of the chromogranin A protein family. *Biochem. J.* **262,** 1–13

40. Lazure, C., Paquet, L., Litthauer, D., Naude, R. J., Oelofsen, W., and Chretien, M. (1990) The ostrich pituitary contains a major peptide homologous to mammalian chromogranin A(1–76). *Peptides* **11,** 79–87

41. Turquier, V., Vaudry, H., Jegou, S., and Anouar, Y. (1999) Frog chromogranin A messenger ribonucleic acid encodes three highly conserved peptides. Coordinate regulation of proopiomelanocortin and chromogranin A gene expression in the pars intermedia of the pituitary during background color adaptation. *Endocrinology* **140,** 4104–4112

42. Brekke, J. F., Kirkeleit, J., Lugardon, K., and Helle, K. B. (2000) Vasostatins. Dilators of bovine resistance arteries. *Adv. Exp. Med. Biol.* **482,** 239–246.

43. Brekke, J. F., Osol, G. J., and Helle, K. B. (2002) N–terminal chromogranin–derived peptides as dilators of bovine coronary resistance arteries. *Regul. Pept.* **105,** 93–100.

44. Corti, A., Mannarino, C., Mazza, R., Colombo, B., Longhi, R., and Tota, B. (2002) Vasostatins exert negative inotropism in the working heart of the frog. *Ann N.Y. Acad. Sci.* **971,** 362–365.

45. Mandalà, M., Stridsberg, M., Helle, K. B., and Serck-Hanssen, G. (2000) Endothelial handling of chromogranin A. *Adv. Exp. Med. Biol.* **482,** 167–178

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Table 1

Effect of CgA and CgA fragments on TNF-induced leakage of trypan-blue albumin from liver vessels

	Group	**Treatment**[a]	Number of mice (*n*)	Leakage of dye (Mean ± SD) (A540nm)	Unpaired *t* test *(P value)*	
Exp. 1						
	G1	None	12	350 ± 55		
	G2	TNF	12	1282 ± 291	(G2 vs. G1)	$P < 0.0001$
	G3	TNF + CgA	12	703 ± 68	(G3 vs. G2)	$P < 0.0001$
	G4	CgA	12	342 ± 57	(G4 vs. G1)	*ns*
	G5	TNF + CgA + 5A8	12	1009 ± 149	(G5 vs. G3)	$P < 0.0001$
	G6	TNF + CgA + 19E12	12	682 ± 85	(G6 vs. G3)	*ns*
Exp. 2						
	G1	None	5	301 ± 35		
	G2	TNF	5	973 ± 42	(G2 vs. G1)	$P < 0.0001$
	G3	TNF + CgA_{1-78}	5	447 ± 21	(G3 vs. G2)	$P < 0.0001$
	G4	CgA_{1-78}	5	467 ± 18		
Exp. 3						
	G1	None	3	386 ± 69		
	G2	TNF	3	1045 ± 63	(G2 vs. G1)	$P = 0.0003$
	G3	TNF + CgA_{1-78}	3	670 ± 126	(G3 vs. G2)	$P = 0.01$
	G4	TNF + CgA_{1-78} + 5A8	3	1009 ± 46	(G4 vs. G3)	$P = 0.012$
	G5	TNF + CgA_{1-78} + 9E10	3	854 ± 79	(G3 vs. G2)	ns
	G6	TNF + 9E10	3	1075 ± 75	(G6 vs. G2)	ns
	G7	TNF + CgA_{7-57}	3	601 ± 10	(G7 vs. G2)	$P = 0.0003$

[a] Murine TNF (2 ng); human CgA (0.7 µg); mAb 5A8, 9E10 and 19E12 (20 µg); CgA_{1-78} and CgA_{7-57} (3 µg).

Fig. 1

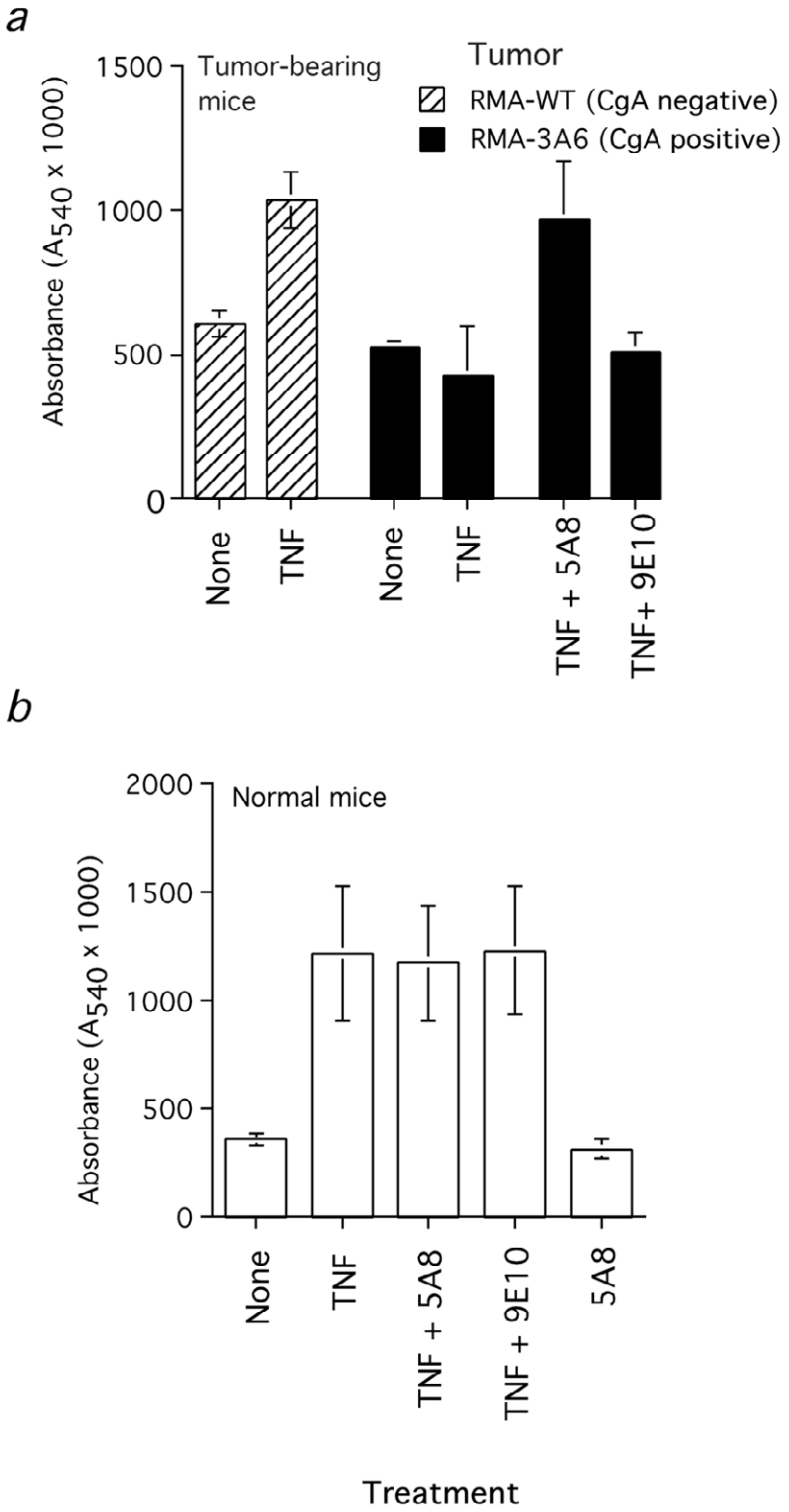

Figure 1. Effect of endogenous CgA and of anti-CgA neutralizing mAb 5A8 on TNF-induced leakage of trypan blue-albumin from liver vessels in (***a***) tumor-bearing mice and (***b***) in normal mice**.** Normal BALB/c mice and CD-1 nu/nu BR mice bearing CgA-secreting (RMA-3A6) and CgA nonsecreting (RMA-WT) tumors were injected intraperitoneally with the reagents indicated below each panel, 30 min before liver perfusion with trypan-blue albumin as described in "Materials and Methods". The following doses were used: TNF (2 ng), anti-CgA mAb 5A8 (30 µg), or control mAb 9E10 (30 ng) in sterile 0.9% sodium chloride. Controls ("None") received 0.9% sodium chloride. Each bar represents the mean ± SD of three mice.

Fig. 2

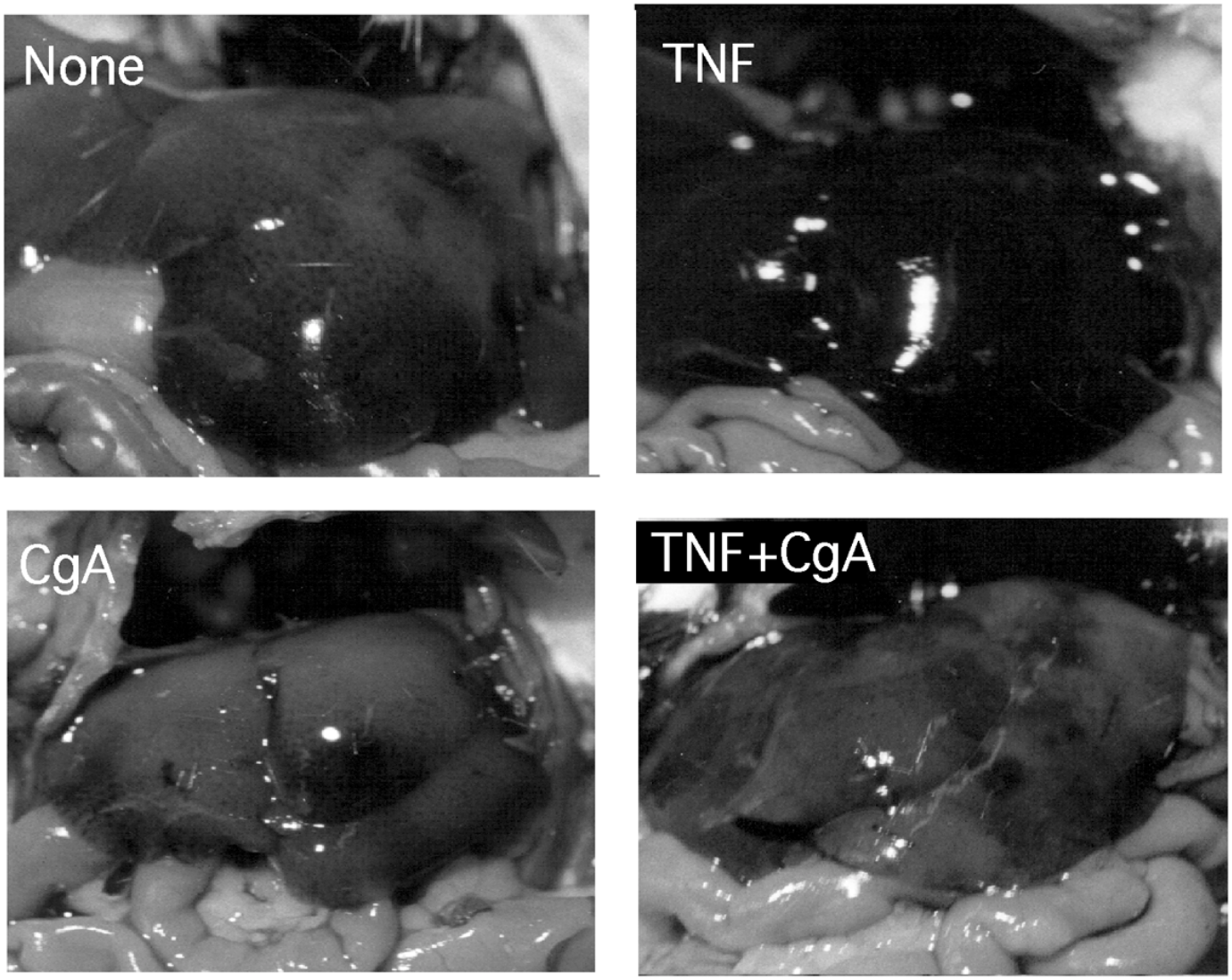

Figure 2. Effect of exogenous human CgA on TNF-induced leakage of trypan blue-albumin from liver vessels of mice. Mice were injected intraperitoneally with the reagents indicated in each panel, 30 min before liver perfusion with trypan-blue albumin. Representative photographs of livers after treatment as described for "Exp. 1" of **Table 1**. The following dose of each reagent was injected: murine TNF (2 ng); CgA (0.7 μg).

Fig. 3

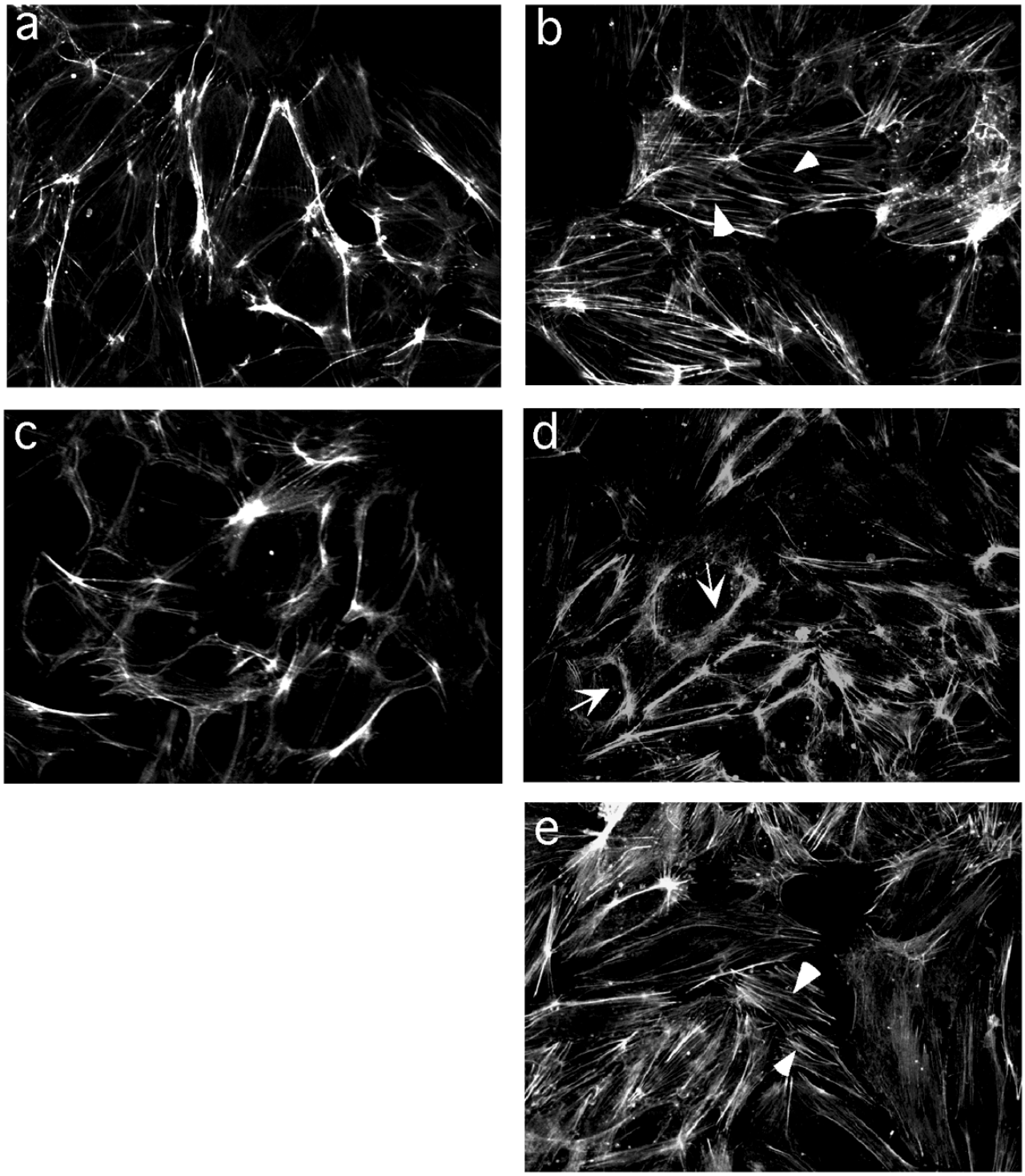

Figure 3. Effect of CgA1-78 on TNF-induced cytoskeletal reorganization of HUVEC. Confocal microscopy of FITC-phalloidin-stained HUVEC preincubated for 2 h with culture medium alone (***a, c***) or medium containing 4 ng/ml human TNF (***b, d, e***), in the absence (***a, b***) or in the presence (***c, d***) of CgA1-78 (330 nM). (***a***) A control experiment is shown in which neither TNF nor CgA1-78 was added. (***e***) The effect of a mixture of TNF (4 ng/ml), CgA1-78 (330 nM), and mAb 5A8 (330 nM) is shown. Arrows indicate F-actin peripheral bundles. Arrowheads indicate stress fibers.

Fig. 4

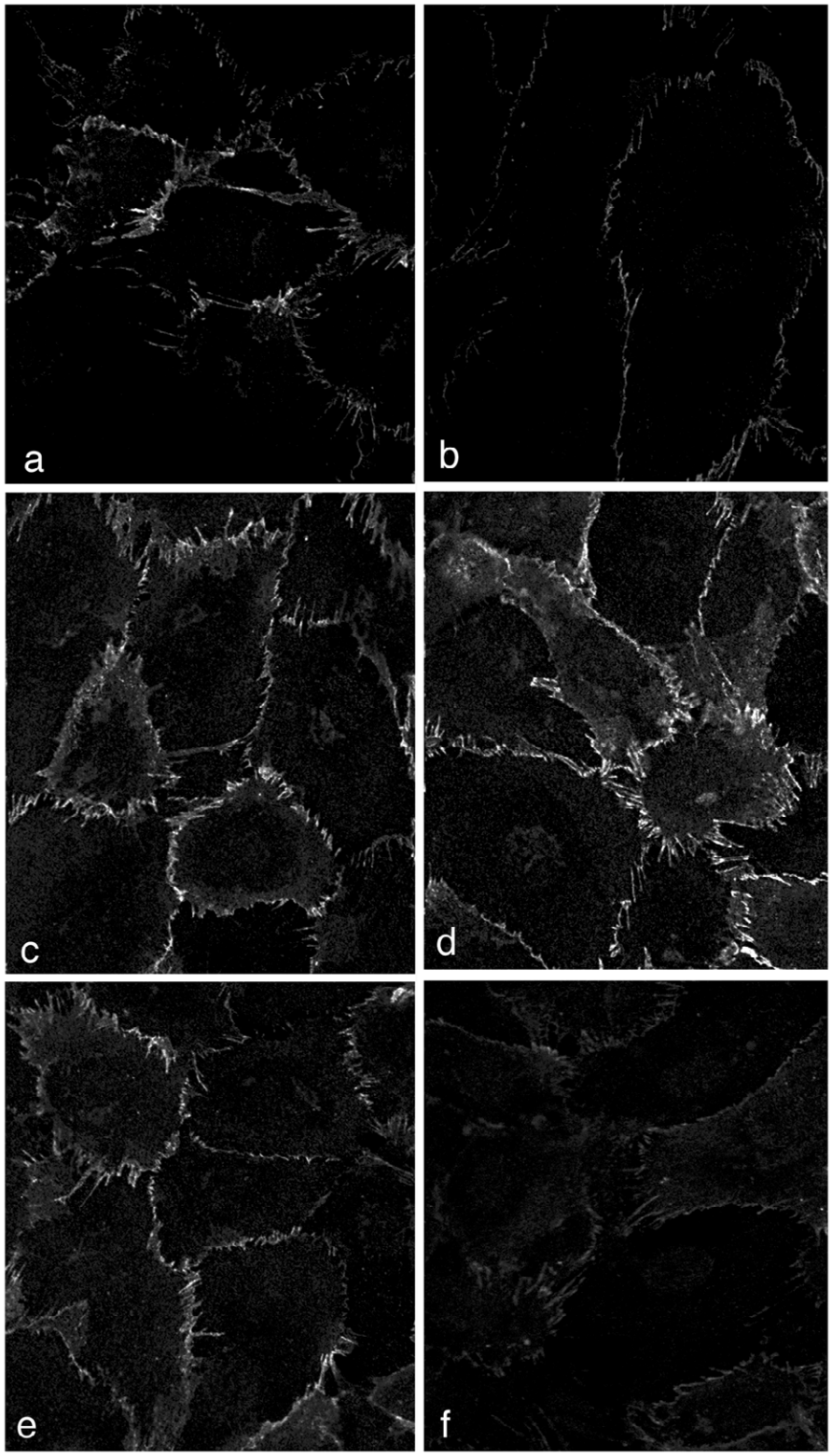

Figure 4. Effect of CgA1-78 and TNF on VE-cadherin expression by HUVEC. Expression of VE-cadherin was evaluated by confocal microscopy as described in "Material and Methods". Before staining, the cells were preincubated for 2 h with culture medium alone (***a, c, e***) or medium containing 4 ng/ml human TNF (***b, d, f***) in the absence or in the presence of CgA1-78. (***a*** and ***b***) without CgA1-78; (***c*** and ***d***) with 330 nM CgA1-78. (***e*** and ***f***): with CgA1-78 (330 nM) and mAb 5A8 (330 nM).

Fig. 5

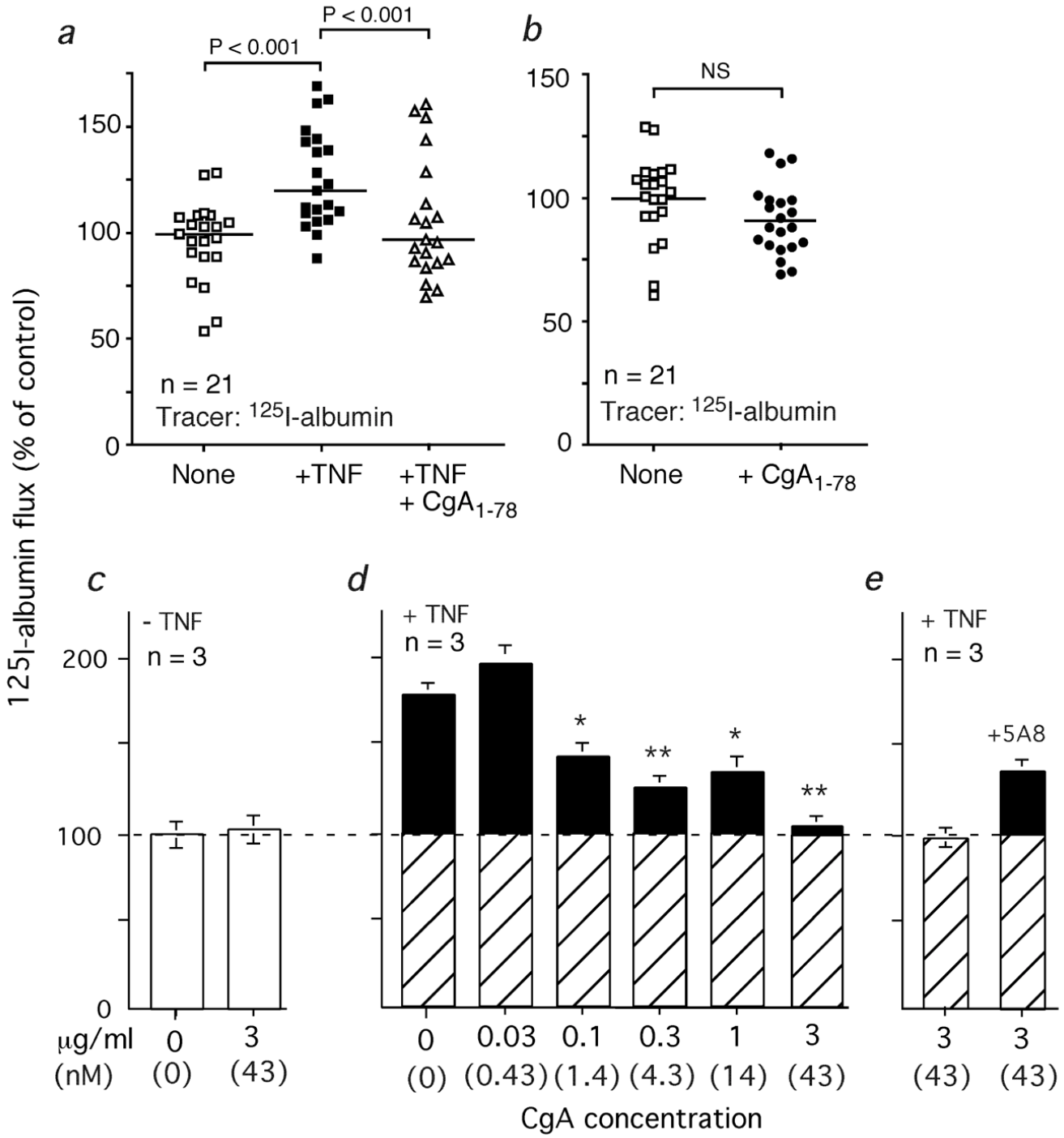

Figure 5. Effect of CgA on TNF-induced permeability of cultured HUVEC monolayers. The assay was carried out with cells untreated or treated with 4 ng/ml human TNF, in the presence of 330 nM CgA1-78 (***a, b***) or various doses of CgA (***c–e***). The effect of CgA and TNF was measured also in the presence of the anti-CgA mAb 5A8 (200 nM) (***e***). The (Y) axis reports the ^{125}I-albumin present in the lower chamber of the Transwell systems after 1 h, expressed as a percentage of cells treated with medium alone. Horizontal bars (***a*** and ***b***) represent the mean values of 7 independent experiments (each in triplicate) carried out with HUVEC from different donors. Dashed bars (***d*** and ***e***) represent the basal permeability in the absence of TNF; black bars represent the TNF-induced permeability. (*) P<0.05; (**) P< 0.01 (cells treated with TNF vs. CgA/TNF mixtures).

Fig. 6

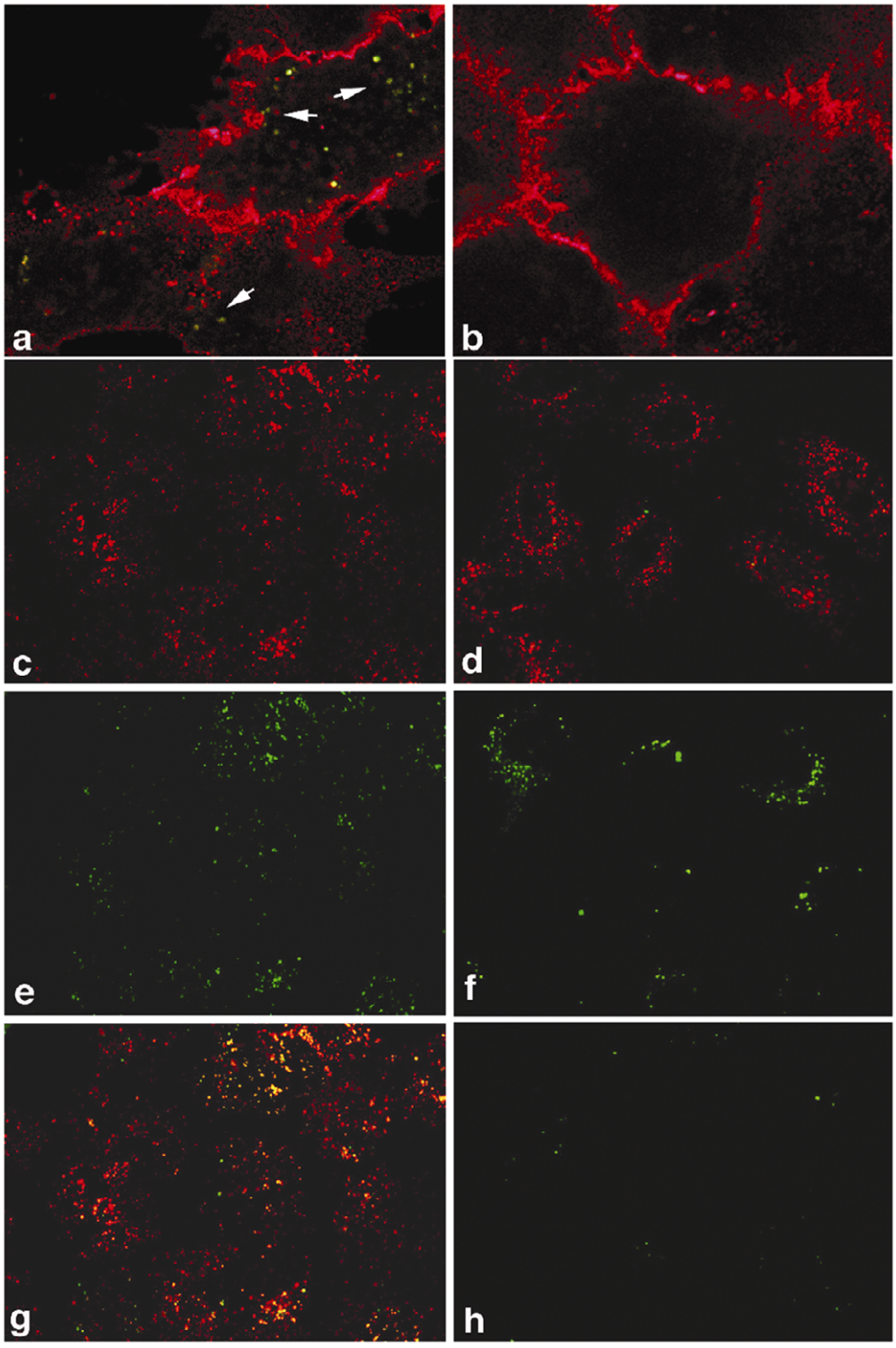

Figure 6. Internalization of CgA1-78 by endothelial cells. Confocal microscopy of HUVEC after incubation with 10 µg/ml FITC-CgA1-78 (***a***) or 10 µg/ml FITC-streptavidin (***b***) for 4 h at 37°C; (***a*** and ***b***) The cells were counterstained with mAb M89D3 (anti-CD31) and TRITC-labeled rabbit anti-mouse immunoglobulin (red). Arrows indicate FITC-CgA1-78 (green). Confocal microscopy of HUVEC incubated with 100 µg/ml Alexa 568-transferrin conjugate (Molecular Probes) (***d***), or with 10 µg/ml FITC-CgA1-78 (***f***), or with culture medium alone (***h***) or with both compounds (***c*** and ***e***). (***c***, ***e,*** and ***g***) The same field is shown as it appears using TRITC (***c***) or FITC (***e***) filters, or by computer merging of the two images (***g***).

Fig. 7

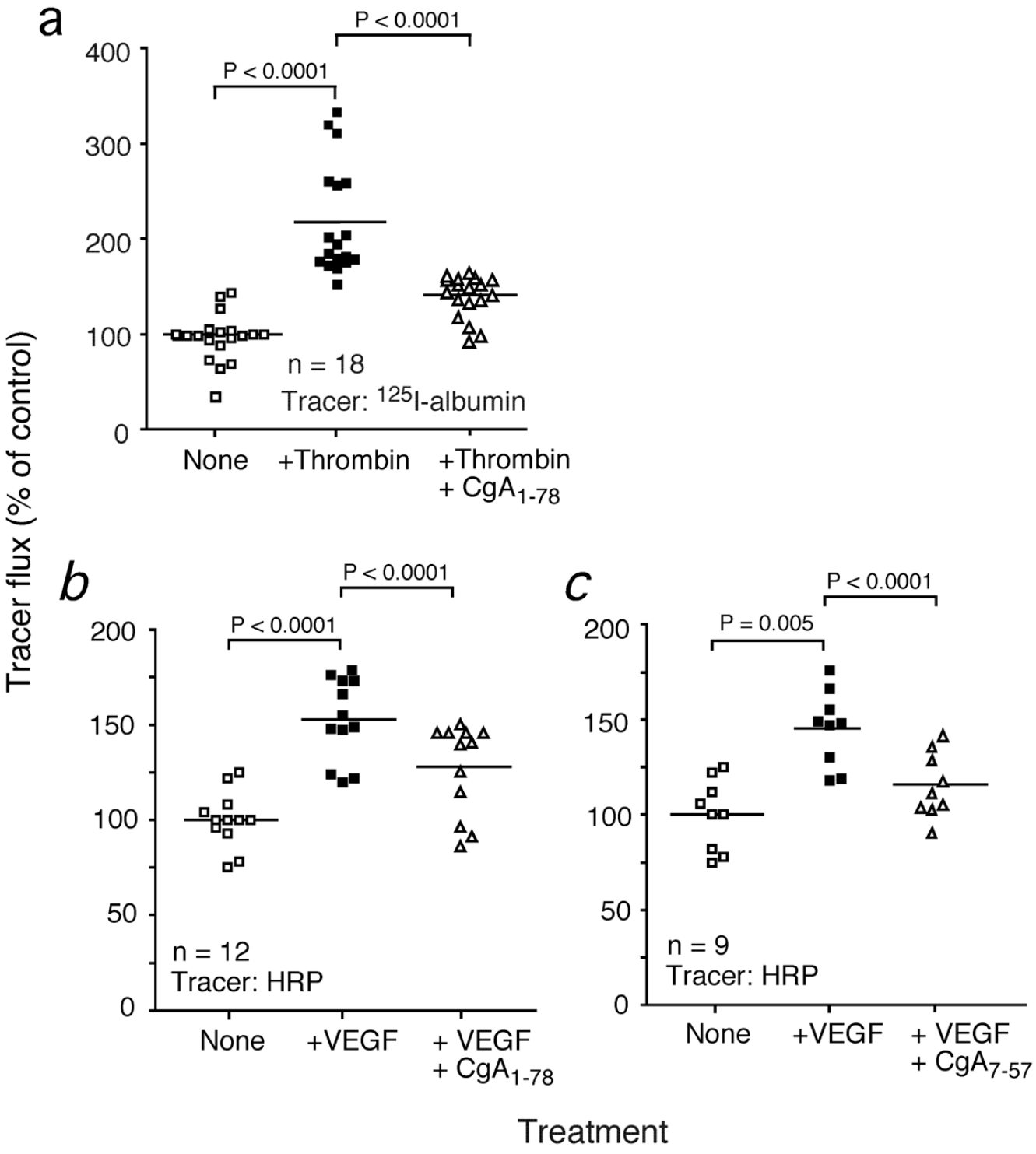

Figure 7. Effect of CgA on thrombin (*a*) and VEGF-induced (*b, c*) permeability of cultured HUVEC monolayers. The cells were treated with 2 U/ml thrombin or 20 ng/ml VEGF or culture medium alone (none), with or without 330 nM CgA1-78 or CgA7-57 as indicated. The (Y) axis reports the amount of tracer (^{125}I-albumin or HRP) present in the lower chamber of the Transwell systems after 1 h, expressed as a percentage of cells treated with medium alone. Horizontal bars (***a*** and ***b***) represent the mean values 6 (*a*), 4 (*b*) and 3 (*c*) independent experiments (each in triplicate) carried out with HUVEC from different donors. Statistical analysis was performed by unpaired *t*-test, two-tailed.