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Short communication

Antithrombin increases pulmonary endothelins: inhibition by heparin and Ca^{2+} channel antagonism

Karl Stangl [a,*], Thomas Dschietzig [a], Konstantin Alexiou [a], Friedrich Brunner [b]

[a] *Medizinische Klinik und Poliklinik I, Charité, Humboldt-Universität zu Berlin, Schumannstrasse 20 / 21, D-10098 Berlin, Germany*
[b] *Institut für Pharmakologie und Toxikologie, Karl-Franzens-Universität Graz, Universitätsplatz 2, A-8010 Graz, Austria*

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Abstract

We investigated the mechanism of antithrombin III-induced vascular release of endothelins in rat isolated lung. The antithrombin III-stimulated release of big endothelin-1 and endothelin-1 (1.7-fold and 1.3-fold over baseline) was abolished by nicardipine (L-type Ca^{2+} channel blocker), heparin, and *N*-acetyl heparin (a derivative devoid of antithrombin affinity), whereas staurosporine and genistein (inhibitors of protein kinase C and tyrosine kinase, respectively) were ineffective. Thus, (i) the antithrombin III-induced release of endothelins requires extracellular Ca^{2+}, but not protein kinase C or tyrosine kinase activation, and (ii) heparin binding to antithrombin III is not necessary for its inhibitory effect. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Antithrombin III; Endothelin; Ca^{2+} channel, L-type; Protein kinase; Lung, rat

1. Introduction

Antithrombin III, a plasma glycoprotein with a molecular weight of 65 kDa, is an inhibitor of serine proteases. Its main physiological function is inactivation of the key clotting enzyme, thrombin, but it also inactivates other clotting and clot-lysing enzymes which are serine proteases. The anticoagulant, heparin, binds to antithrombin III and thereby greatly accelerates the rate of inactivation of proteases by antithrombin III. In vivo, binding to heparin-like glycosaminoglycans appears to be involved in antithrombin III action (Mammen, 1998).

The application of antithrombin III in sepsis and sepsis-related adult respiratory distress syndrome has attracted particular interest due to its effectiveness in reducing mortality in experimental studies (Dickneite and Paques, 1993). In endotoxin-treated rats, antithrombin III attenuated the pulmonary accumulation of leukocytes and the increase in vascular permeability during the course of adult respiratory distress syndrome (Uchiba et al., 1996). Not only is antithrombin III a powerful inhibitor of the clotting system, but is has a protective effect which is generally attributed to prostacyclin-mediated suppression of leukocytes (Kainoh et al., 1990), because antithrombin III stimulates the synthesis of prostacyclin in human and bovine non-pulmonary endothelial cells via heparin-like glycosaminoglycan receptors (Yamauchi et al., 1989). The attenuation of pulmonary damage by antithrombin III is counteracted by the anticoagulant, heparin (Uchiba et al., 1996).

Interestingly, however, clinical studies have so far failed to confirm a reduction of mortality by antithrombin III in sepsis and sepsis-related adult respiratory distress syndrome (Fourier et al., 1993). We have recently found that the administration of antithrombin III in isolated perfused rat lungs stimulates the production of big endothelin-1 and mature endothelin-1 under both basal and septic conditions (unpublished data). This finding is of potential pathophysiological relevance, because up-regulation of pulmonary endothelins by antithrombin III is likely to attenuate the protective effect of the antiprotease, due to the cell-activating, proinflammatory (Battistini et al., 1996) and vasoconstrictor properties of big endothelin-1 and endothelin-1 (Corder and Vane, 1995). Hence, there is much to be gained from identifying the underlying mechanism involved in the unfavourable side-effect of antithrombin III as well as from the development of potential drugs that might alleviate this effect. In the present study, we analyzed the mechanism(s) involved in the antithrombin III-

* Corresponding author. Tel.: +49-30-2802-5885 / +49-30-2802-6373; Fax: +49-30-2802-3996; E-mail: karl.stangl@charite.de

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induced up-regulation of pulmonary endothelins in rat isolated perfused lung and specifically focused on elucidating the possible signal transduction pathways involved. Previous studies have shown the dependence of endothelin-1 secretion on cytosolic Ca^{2+} levels (Brunner, 1995) and on tyrosine phosphorylation (Marsen et al., 1995), as well as an inhibitory action of heparin via a protein kinase C-dependent pathway (Imai et al., 1993). We, therefore, tested the effects of the L-type channel blocker, nicardipine, the tyrosine kinase inhibitor, genistein (Dean et al., 1989), the protein kinase C inhibitor, staurosporine (Matsumoto and Sasaki, 1989), and of heparin on the production of pulmonary endothelins in intact lung.

2. Materials and methods

2.1. Animals and isolated lung preparation

Male Wistar rats, weighing 300 to 400 g, were used for these experiments. The study conforms to the guidelines of the American Heart Association on Research Animal Use adopted on 11 November 1984.

After intraperitoneal anesthesia (thiopental sodium, 40–80 mg/kg body weight [BW]), a tracheotomy permitted positive pressure ventilation with a small animal respirator (60 strokes/min, tidal volume 8–10 ml/kg BW, 1 mm Hg positive end-expiratory pressure, gas mixture 21% O_2, 5% CO_2 and 74% N_2).

A median sternotomy was performed, a cannula was placed into the pulmonary artery, and the heart was removed to allow passive drainage of the pulmonary effluent from the pulmonary veins. Perfusion was carried out with Krebs–Henseleit buffer containing 20 g/l bovine serum albumin (composition in mmol/l: NaCl, 127; KCl, 3.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.1; $NaHCO_3$, 25.0; glucose, 10; pyruvate, 1.8; and *N*-2-hydroxyethylpiperazine-*n*-2-ethanesulfonic acid [HEPES], 5.0; pH = 7.35–7.40 at 37.5°C). The lungs were suspended in a humidified chamber from a force transducer that monitored changes in lung weight. Within 20 min, pulmonary flow was gradually increased to 11 ml/min. Only lungs were selected for the present study that showed a constant pulmonary mean arterial pressure (4–6 mm Hg, zero-referenced at hilus), a constant peak inflation pressure of 7 to 10 mm Hg, no weight gain (< 50 mg/h), and no signs of haemorrhage, edema or atelectasis.

2.2. Experimental protocol

The lungs were perfused for 2 h with 80 ml of buffer in recirculatory mode, the entire perfusate was rapidly frozen in liquid N_2 and stored at −70°C for determination of big endothelin-1 and endothelin-1.

The following drugs were administered in combination with 5 U/ml antithrombin III: (1) nicardipine (50 μM, $n = 7$); (2) heparin (50 μg/ml, $n = 6$; this concentration is maximally effective to block the antithrombin III-induced prostacyclin stimulation in cultured endothelial cells; Yamauchi et al., 1989); (3) *N*-acetyl heparin (50 μg/ml, $n = 6$), a derivative of heparin devoid of affinity for antithrombin III and anticoagulant activity (Friedrichs et al., 1994); (4) staurosporine (50 nM, $n = 8$); 5) genistein (100 μM, $n = 8$). In addition, all drugs were studied in isolated lungs under basal conditions ($n = 4$ for each drug) to document their intrinsic effects on the pulmonary release of big endothelin-1 and endothelin-1. Control lungs were perfused with vehicle and 5 U/ml antithrombin III, respectively ($n = 13$ in both cases).

2.3. Determination of big endothelin-1 and endothelin-1

For determination of big endothelin-1 by radioimmunoassay, a polyclonal antiserum against big endothelin-1 (1–39; rat) was raised in rabbits. Standard solutions of big endothelin-1 (1–39; rat) were used at 2–512 pg/assay; bound radioactivity was separated using polyethylene glycol. The detection limit was ~ 2 pg per assay tube (~ 10% displacement of the radioactive tracer) and the IC_{50} value was 45 pg/tube. Cross-reaction of the antiserum was 16% with big endothelin-1 fragment 22–39 (bovine), and zero with endothelin-1, atrial natriuretic peptide (1–28; rat) and angiotensin II (~ 0.1% at the IC_{50} concentration in each case).

The concentration of endothelin-1 was determined by radioimmunoassay using a commercial kit (Peninsula, Belmont, CA) as described previously (Brunner, 1995). The detection limit was ~ 0.15 pg/tube; the cross-reactivity of other endothelin isomers and big endothelin-1 in this assay was less than 5 and 37%, respectively, according to the supplier.

2.4. Data analysis

The data are presented as means ± S.E.M. Differences between groups were analyzed using the one-way analysis of variance (ANOVA). Individual groups were compared using Dunnett's test. An error probability of $P < 0.05$ was regarded as significant.

2.5. Materials

Nicardipine HCl, heparin sodium, *N*-acetyl heparin sodium, staurosporine, and genistein were purchased from Sigma (Munich, Germany). Antithrombin III (activity, 6.6 IU/mg) was provided by Centeon Pharma (Marburg, Germany).

Drugs were dissolved freshly in perfusion buffer except nicardipine (stock solution of 5 mM in ethanol), staurosporine (stock of 1 mM in dimethyl sulfoxide [DMSO]), and genistein (stock of 10 mM in DMSO). In these cases, the solvent content of the perfusate was 1% or less. All drugs were dissolved at the final concentrations mentioned above.

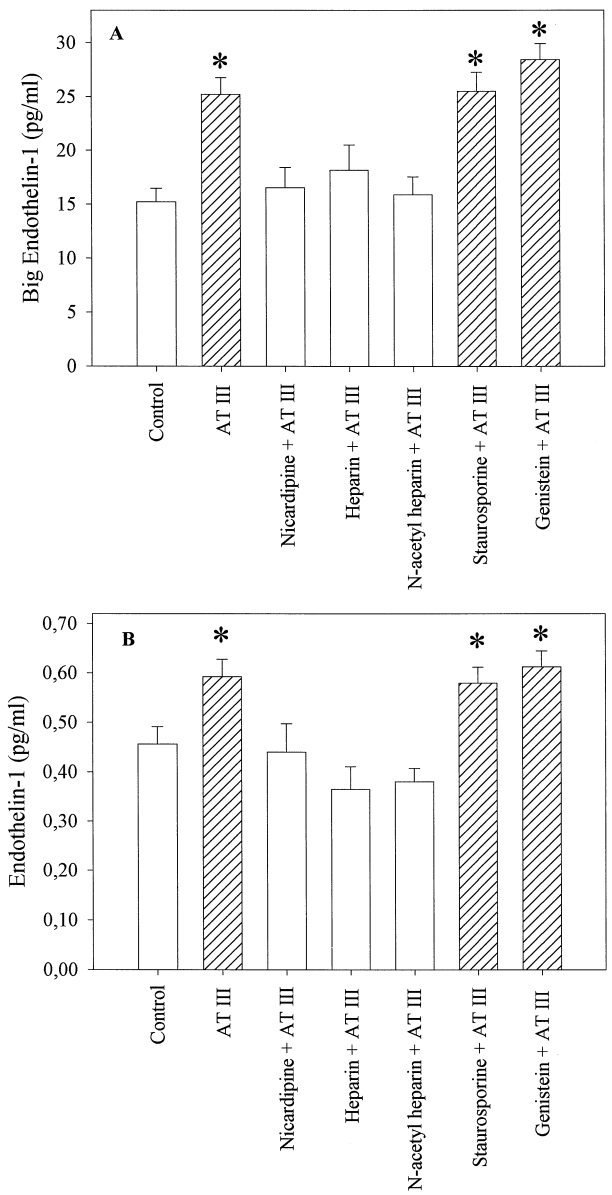

Fig. 1. Release of big endothelin-1 (A) and endothelin-1 (B) in rat lungs perfused with vehicle (controls, $n = 13$), 5 u/ml antithrombin III (AT III) ($n = 13$), or antithrombin III together with nicardipine (50 μM, $n = 7$; L-type Ca^{2+} channel blocker), heparin (50 μg/ml, $n = 6$), *N*-acetyl heparin (50 μg/ml, $n = 6$; a heparin derivative devoid of antithrombin III affinity), staurosporine (50 nM, $n = 8$; an inhibitor of protein kinase C), or genistein (100 μM, $n = 8$; a tyrosine kinase inhibitor). Buffer, 80 ml, was recirculated for 2 h, and big endothelin-1 and endothelin-1 in the perfusate were quantified by radioimmunoassay (detection limits, ~2 pg/tube, big endothelin-1; 0.15 pg/tube, endothelin-1). $* P < 0.05$ vs. control.

3. Results

3.1. Drug effects in the absence of antithrombin III

None of the drugs used affected pulmonary haemodynamics or vascular permeability, as indicated by constant mean arterial pressure and lung weight, respectively. Similarly, the drugs had no effect on the release of big endothelin-1 and endothelin-1. In control lungs, the basal release of big endothelin-1 was 15.2 ± 1.2 pg/ml and of endothelin-1 0.46 ± 0.04 pg/ml. In the presence of nicardipine, the levels were 17.4 ± 3.3 and 0.42 ± 0.04 pg/ml, of heparin, 14.0 ± 2.1 and 0.38 ± 0.04 pg/ml, of *N*-acetyl heparin, 12.0 ± 0.7 and 0.38 ± 0.02 pg/ml, of staurosporine, 16.9 ± 1.6 and 0.50 ± 0.04 pg/ml, and of genistein, 17.5 ± 2.0 and 0.51 ± 0.06 pg/ml.

3.2. Drug effects on antithrombin III-induced increase in big endothelin-1 and endothelin-1 levels

The effect of big endothelin-1 and endothelin-1 of antithrombin III and of the different treatments on pulmonary vascular release is depicted in Fig. 1a and b. Antithrombin III, at a concentration of 5 U/ml, significantly elevated the release of big endothelin-1 (1.7-fold) and of endothelin-1 (1.3-fold; $P < 0.05$ for both). Treatment with nicardipine, heparin and *N*-acetyl heparin abolished the effect of antithrombin III, but staurosporine and genistein were without effect.

4. Discussion

The present study investigated possible mechanisms by which the antiprotease antithrombin III may promote the pulmonary vascular release of big endothelin-1 and endothelin-1. We found that blockade of voltage-dependent Ca^{2+} channels with nicardipine prevented the antithrombin III-induced stimulation, which is consistent with previous findings of a stimulatory effect of intracellular Ca^{2+} on endothelin-1 secretion in cultured endothelial cells (Brunner, 1995). In previous studies with cultured endothelial cells obtained from different conduit vessels, most authors have found no evidence for functional voltage-gated Ca^{2+} channels. However, the release of endothelin-1 in conditioned media of endothelial cells derived from human umbilical artery and vein was inhibited concentration-dependently by nisoldipine, another L-type Ca^{2+} channel blocker (Liu et al., 1993). The present data clearly show that the effect of antithrombin III on endothelin-1 and big endothelin-1 release in the isolated pulmonary vasculature is also mediated by functional voltage-dependent Ca^{2+} channels.

In contrast to Imai et al. (1993) and Marsen et al. (1995), we were not able to document any effect of the

potent protein kinase C inhibitor, staurosporine and of the tyrosine kinase inhibitor, genistein, on basal or antithrombin III-stimulated pulmonary release of big endothelin-1 and endothelin-1. Only one concentration, rather than a range of inhibitor concentrations, was used in the present study to identify underlying mechanisms. This might appear insufficient to allow firm conclusions to be drawn. However, it is unlikely that the concentrations used (staurosporine, 50 nM; genistein, 100 μM) were too low to inhibit protein kinase C or tyrosine kinase signalling substantially, because the affinities for staurosporine and genistein were reported to be in the low nanomolar (K_i = 0.7 nM, Matsumoto and Sasaki, 1989) and in the micromolar range (IC_{50} = 10 to 20 μM, Dean et al., 1989), respectively. Clearly, the application of higher inhibitor concentrations seemed inappropriate, because higher concentrations of both staurosporine and genistein have side-effects, e.g., inhibition by staurosporine of protein kinases A (K_i = 7 nM) and G (K_i = 8.5 nM) (Matsumoto and Sasaki, 1989) and blockade by genistein of Ca^{2+} channels (IC_{50} = 50 μM, Kusaka and Sperelakis, 1995). Species differences, as well as differences inherent to the use of different vascular beds and models (intact organ vs. cultured cells) may account for the divergent observations.

The anticoagulant, heparin and its derivative, *N*-acetyl heparin, which lacks antithrombin III affinity and anticoagulant activity, did not influence the basal secretion of big endothelin-1 or endothelin-1, but prevented the antithrombin III-induced rise of both peptides. It is generally accepted that antithrombin III acts via endothelial heparin-like glycosaminoglycans, because using modified antithrombin III that cannot bind to glycosaminoglycans prevents its effect (Uchiba et al., 1996). Therefore, binding to endothelial glycosaminoglycan receptors appears essential for the effect of antithrombin III. By using heparin and its derivative *N*-acetyl heparin that neither binds to antithrombin III nor potentiates its anticoagulant action, we addressed the question of whether the inhibitory action of heparin vis-à-vis antithrombin III is due to competition with such endothelial receptors or to interference with intracellular pathways of antithrombin III. Our finding that the antithrombin III effect is blocked similarly by antithrombin III-binding and antithrombin III-non-binding heparins favours the latter explanation, i.e., that heparin appears to interact with antithrombin III intracellular signalling rather than with receptor binding. In clinical practice, the co-application of antithrombin III and of the anticoagulant heparin during sepsis is controversial, because the advantage of this combination over antithrombin III given alone has never been examined in controlled studies and, furthermore, heparinization will inevitably exacerbate any bleeding tendency (Mammen, 1998). In the light of these considerations, the administration of heparin derivatives devoid of anticoagulant activity but that prevent the unfavourable antithrombin III effect of stimulating pulmonary endothelins might prove useful.

In conclusion, the stimulatory effect of antithrombin III on pulmonary big endothelin-1 and endothelin-1 is dependent on Ca^{2+} influx through functional voltage-gated Ca^{2+} channels, and the inhibition by heparin does not require direct binding of antithrombin III. Both the stimulatory effect of antithrombin III and the inhibitory effect of heparins appear not to involve protein kinase C or tyrosine kinase signalling in intact rat lung. Finally, the identification of a Ca^{2+} entry blocker and a heparin that does not bind antithrombin III as inhibitors of the antithrombin III-induced stimulation of pulmonary endothelins may be of clinical relevance in the treatment of sepsis with this antiprotease.

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References

Battistini, B., Forget, M.A., Laight, D., 1996. Potential roles for endothelins in systemic inflammatory response syndrome with particular relationship to cytokines. Shock 5, 176–183.

Brunner, F., 1995. Dependence of endothelin-1 secretion on Ca^{2+}. Biochem. Pharmacol. 49, 1785–1791.

Corder, R., Vane, J.R., 1995. Radioimmunoassay evidence that the pressor effect of big endothelin-1 is due to local conversion to endothelin-1. Biochem. Pharmacol. 49, 375–380.

Dean, N.M., Kanemitsu, M., Boynton, A.L., 1989. Effects of the tyrosine-kinase inhibitor genistein on DNA synthesis and phospholipid-derived second messenger generation in mouse 10T1/2 fibroblasts and rat liver T51B cells. Biochem. Biophys. Res. Commun. 165, 795–801.

Dickneite, G., Paques, E.P., 1993. Reduction of mortality with antithrombin III in septicemic rats: a study of *Klebsiella pneumoniae* induced sepsis. Thromb. Haemost. 69, 98–102.

Fourier, F., Chopin, C., Huart, J.J., Runge, I., Caron, C., Goudemand, J., 1993. Double-blind, placebo-controlled trial of antithrombin III concentrates in septic shock with disseminated intravascular coagulation. Chest 104, 882–888.

Friedrichs, G.S., Kilgore, K.S., Manley, P.J., Gralinski, M.R., Luccesi, M.R., 1994. Effects of heparin and *N*-acetyl heparin on ischemia/reperfusion-induced alterations in myocardial function in the rabbit isolated heart. Circ. Res. 75, 701–710.

Imai, T., Hirata, Y., Emori, T., Marumo, F., 1993. Heparin has an inhibitory effect on endothelin-1 synthesis and release by endothelial cells. Hypertension 21, 353–358.

Kainoh, M., Imai, R., Umetsu, T., Hattori, M., Nishio, S., 1990. Prostacyclin and beraprost sodium as suppressors of activated rat polymorphnuclear leukocytes. Biochem. Pharmacol. 39, 477–484.

Kusaka, M., Sperelakis, N., 1995. Inhibition of L-type calcium current by genistein, a tyrosine kinase inhibitor, in pregnant rat myometrial cells. Biochim. Biophys. Acta 1240, 196–200.

Liu, J.J., Casley, D., Wojta, J., Gallicchio, M., Dauer, R., Buxton, B.F., Johnston, C.I., 1993. Reduction of endothelin levels by the dihydropyridine calcium antagonist nisoldipine and a natural factor in cultured human endothelial cells. J. Hypertens. 11, 977–982.

Mammen, E.F., 1998. Antithrombin: its physiological importance and role in DIC. Semin. Thromb. Haemost. 24, 19–24.

Marsen, T.A., Simonson, M.S., Dunn, M.J., 1995. Thrombin induces the preproendothelin-1 gene in endothelial cells by a protein tyrosine kinase-linked mechanism. Circ. Res. 76, 987–995.

Matsumoto, H., Sasaki, Y., 1989. Staurosporine, a protein kinase C inhibitor, interferes with proliferation of arterial smooth muscle cells. Biochem. Biophys. Res. Commun. 158, 105–109.

Uchiba, M., Okajima, K., Murakami, K., Okabe, H., Takatsuki, K., 1996. Attenuation of endotoxin-induced pulmonary injury by antithrombin III. Am. J. Physiol. 270, L921–L930.

Yamauchi, T., Umeda, F., Inoguchi, T., Nawata, H., 1989. Antithrombin III stimulates prostacyclin production by cultured aortic endothelial cells. Biochem. Biophys. Res. Comm. 29, 1404–1411.