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European Journal of Pharmacology 427 (2001) 115–118

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Platelet-induced inhibition of adrenomedullin secretion

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Received 11 July 2001; received in revised form 27 July 2001; accepted 1 August 2001

Abstract

Adrenomedullin is a biologically active peptide released from the vascular wall, which increases blood flow through its vasorelaxant effects and prevents platelet activation by stimulation of nitric oxide synthesis. The present study demonstrates that activated platelets suppress adrenomedullin secretion from vascular endothelial cells by releasing a factor that was identified as transforming growth factor (TGF)-β1. Adrenomedullin levels were reduced by up to 40% and this effect was completely abrogated by the addition of latency-associated protein (LAP) or TGF-β1-neutralizing antibody. Inhibition of adrenomedullin secretion in response to platelet aggregation may be an important mechanism in the induction of hemostasis.

Keywords: Adrenomedullin; Platelet; TGF-β1 (transforming growth factor-β1); Latency-associated protein

1. Introduction

Adrenomedullin is a 52-amino acid peptide that was originally identified and isolated from human pheochromocytoma by monitoring its activity to induce cAMP synthesis in rat platelets (Kitamura et al., 1993). In addition to the adrenal medulla, the peptide is produced by a number of other tissues including cells of the vascular wall such as endothelial and vascular smooth muscle cells (Sugo et al., 1994). Vascular endothelial cells exhibit a particularly high abundance of adrenomedullin mRNA and represent the major source of circulating adrenomedullin. The endothelial-derived peptide also acts in an auto/paracrine fashion and has been shown to stimulate nitric oxide synthesis in endothelial cells, and decrease vascular tone by activation of adenylate cyclase activity in smooth muscle cells (Shimekake et al., 1995). The interaction between platelets and the vascular wall plays a crucial role in hemostasis and thrombosis, and adrenomedullin may be an important mediator of this. The aim of the present study was to assess the role of platelets in the regulation of adrenomedullin secretion by vascular endothelial cells and the possible effect of adrenomedullin on human platelet aggregation.

2. Materials and methods

2.1. Preparation of platelet supernatant

Human platelet-rich plasma was centrifuged at $600 \times g$ for 15 min. The platelets were washed in 12 mmol Tris–HCl buffer containing 139 mmol/l NaCl and centrifuged again at $600 \times g$ for 10 min. The pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 0.5% fetal calf serum and 0.3 U/ml thrombin. After 4 h of incubation, the platelets were removed by centrifugation at $2000 \times g$ for 15 min. The conditioned medium was heated for 10 min at 80 °C and after cooling, was immediately used for experiments in different concentrations.

Vascular endothelial cells were isolated from bovine thoracic aortae as previously reported (Jaffe et al., 1978). The cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum and used at passages 12–16. The cells were washed twice with serum-free DMEM and maintained in DMEM containing 0.5% fetal calf serum for 24 h before the experiments. To study the release of adrenomedullin, media from cells were replaced by fresh DMEM containing 0.5% fetal calf serum. After 24 h of incubation at 37 °C in a CO_2 (5%) incubator, cell media were aspirated off, centrifuged for 10 min at $250 \times g$ and used for radioimmunoassay. In some experiments, latency-associated pro-

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)01269-9

tein (LAP, R and D) or transforming growth factor (TGF)-β-neutralizing antibody (R and D) was added to neutralize platelet-derived TGF-β. We also stimulated endothelial cells with recombinant TGF-β (Serva) in concentrations ranging from 0.001 to 10 ng/ml DMEM containing 5% fetal calf serum. Conditioned media were treated as described above.

The concentration of adrenomedullin in endothelial cell supernatants was measured without extraction by radioimmunoassay as described (Ehlenz et al., 1997). The cross-reactivity of the assay with calcitonin gene-related peptide was less than 0.5% on a molar basis. Standards were diluted in fresh culture medium to offset potential nonspecific effects of sample media on radioimmunoassay.

Aggregation measurements by following light scattering changes were carried out in platelet-rich plasma in an aggregometer. Aggregation was induced by thrombin (0.15 U/ml) with and without pretreatment of the platelets with adrenomedullin (10^{-5} M) or prostaglandin I_2 (10^{-8} M).

2.2. Statistics

Values are expressed as means ± S.D. Statistical analysis of data was performed using one-way analysis of variance and Student's *t*-test.

3. Results

Incubation of endothelial cells with the supernatant of thrombin-activated platelets resulted in a 37% reduction of the adrenomedullin concentration in the medium, as compared to untreated cells (Fig. 1). To determine the possible role of TGF-β1 in the mediation of this effect, latency-associated protein (LAP), which inactivates TGF-β1 by formation of a high-molecular-weight complex, was added to the platelet supernatant before incubation with endothelial cells. The platelet-induced decrease of adrenomedullin was completely abrogated by LAP. The same effect was observed, following the addition of a TGF-β1-neutralizing antibody (data not shown). Interestingly, in control experiments, incubation of endothelial cells with LAP or TGF-β1-neutralizing antibody alone caused a small but consistent increase of adrenomedullin in the endothelial cell media.

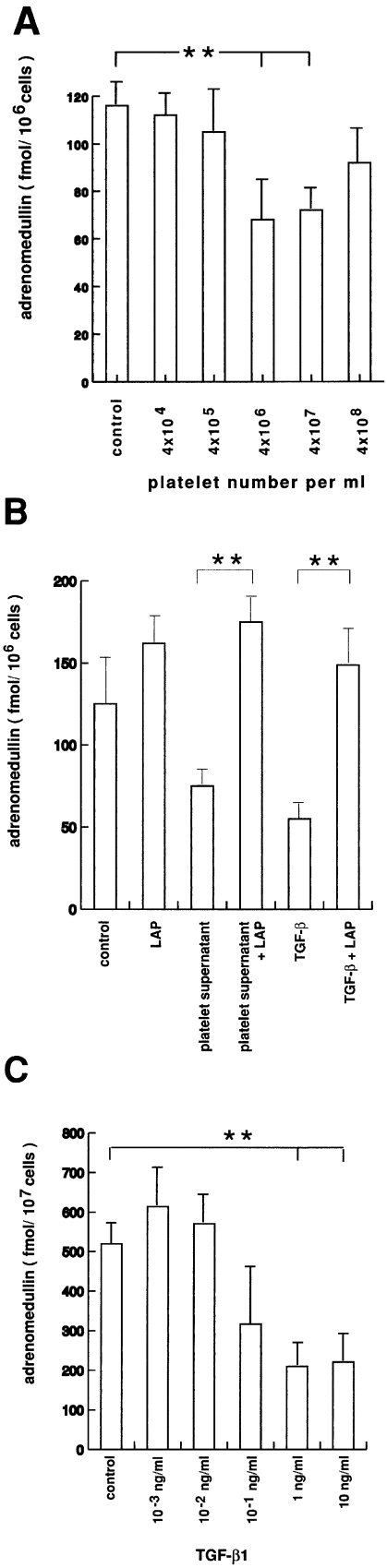

Fig. 1. Effect of thrombin-activated platelet supernatant or recombinant TGF-β on adrenomedullin release from bovine endothelial cells. (A): effect of platelet concentration on adrenomedullin secretion by endothelial cells. Control: stimulation with unconditioned medium (mean ± S.D., $n = 12$, $^{**}P \leq 0.01$). (B): effect of TGF-β neutralization by latency associated protein (LAP) on platelet supernatant or TGF-ß induced adrenomedullin suppression (mean ± S.D., $n = 4$, $^{**}P \leq 0.01$). (C): dose–response relationship between recombinant TGF-β and adrenomedullin release from cultured endothelial cells (mean ± S.D., $n = 11$, $^{**}P \leq 0.01$).

To directly assess the effect of TGF-β1 on adrenomedullin secretion, endothelial cells were stimulated with increasing concentrations of recombinant TGF-β1. A U-shaped concentration-dependent decrease of the adrenomedullin levels with a maximum of 61% at a TGF-β concentration of 1 ng/ml was observed (Fig. 1). Again, this effect was completely abolished by the addition of LAP or TGF-β1 antibody.

In order to address the question whether adrenomedullin affects platelet activity, reversible aggregation of human platelets was induced by the addition of 0.15 U/ml thrombin in the presence or absence of adrenomedullin. Pretreatment of platelets with adrenomedullin had no effect on aggregation, even at low adrenomedullin concentrations (10^{-5} M). Prostaglandin I_2, which was used as reference compound, completely inhibited thrombin-induced aggregation (data not shown).

4. Discussion

Our study demonstrates that platelet aggregation is associated with the release of a factor capable of inhibiting adrenomedullin secretion from vascular endothelial cells. We found that among the numerous bioactive substances released upon platelet activation, TGF-β1 may be crucial in the mediation of this inhibitory effect. This is indicated by the observation that the addition of a TGF-β-neutralizing antibody or LAP completely abrogated the platelet-induced decrease in adrenomedullin secretion. LAP very efficiently blocks TGF-β activity by complex-formation (Bottinger et al., 1996). Interestingly, addition of LAP or antibody alone to endothelial cells slightly increased adrenomedullin release, indicating the presence of TGF-β1 in the media of untreated cells. In fact, as a confirmation of previous studies, we were able to detect TGF-β1 in our endothelial cell media, although only in its inactive form (Ribeiro et al., 1995). We found an average concentration of TGF-β in activated platelet supernatants of 10.8 ng/ml, which was not significantly altered by acidification. In contrast, active TGF-β1 levels were below the detection limit in endothelial cell media, but rose to 1.3 ng/ml following acidification. This indicates the presence of inactive TGF-β1 in the endothelial cell media. Activation may occur through the action of proteases of endothelial origin or the proteolytic activity of the fetal calf serum contained in the media.

Inhibition of adrenomedullin release from endothelial cells by activated platelets might play an important role in primary hemostasis. Following disruption of the vascular endothelial lining, platelet adhere to the subendothelial connective tissue and release their preformed granule constituents. Among these are mediators of aggregation, growth factors for the induction of vascular repair such as TGF-β and a number of vasoconstrictors that stop hemorrhage by reducing perfusion pressure (Assoian and Sporn, 1986). Suppression of adrenomedullin secretion from intact endothelial cells surrounding the lesion or located opposite to it should complement these actions. Elimination of the stimulatory effect of AM on nitric oxide synthesis will promote platelet aggregation and blood vessel constriction (Radomski et al., 1987). In addition, this will reduce the direct relaxatory effects of adrenomedullin on vascular smooth muscle cells, and in the longer term, the inhibition of cell migration by adrenomedullin (Horio et al., 1995).

Adrenomedullin was originally discovered by monitoring the stimulating effect of pheochromocytoma extracts on cAMP levels in rat platelets (Kitamura et al., 1993). It is well known that an increase in intracellular cAMP inhibits platelet activation and aggregation. Therefore, we examined in further experiments the possible influence of adrenomedullin on the function of human platelets. No direct effect of adrenomedullin on thrombin-induced platelet aggregation was noticed, even when high adrenomedullin concentrations were used. This confirms a previous study, which reported that adrenomedullin does not alter the aggregatory response of human platelets to ADP, due probably to the lack of functional adrenomedullin receptors (Schiller et al., 1998). Nevertheless, adrenomedullin is very likely to inhibit platelet aggregation in vivo as it stimulates nitric oxide release by endothelial cells, and nitric oxide is a very potent blocker of platelet aggregation (Shimekake et al., 1995; Radomski et al., 1987).

Our results demonstrate that adrenomedullin secretion is inhibited by platelet-derived factors and that TGF-β1 plays an essential role in the mediation of this effect. This mechanism may also be operative in vivo and thus be part of the complex response to vascular lesions, which relies on the delicate balance between activation of factors promoting hemostasis and wound repair, and suppression of those opposing these effects such as adrenomedullin.

References

Assoian, R.K., Sporn, M.B., 1986. Type beta transforming growth factor in human platelets: release during platelet degranulation and action on vascular smooth muscle cells. J. Cell Biol. 102, 1217–1223.

Bottinger, E.P., Factor, V.M., Tsang, M.L., Weatherbee, J.A., Kopp, J.B., Quian, S.W., Wakefield, L.M., Roberts, A.B., Thorgeirsson, S.S., Sporn, M.B., 1996. The recombinant proregion of transforming growth factor beta1 (latency-associated peptide) inhibits active transforming growth factor beta1 in transgenic mice. Proc. Natl. Acad. Sci. U. S. A. 93, 5877–5882.

Ehlenz, K., Koch, B., Preuss, P., Simon, B., Koop, I., Lang, R.E., 1997. High levels of circulating adrenomedullin in severe illness: correlation with C-reactive protein and evidence against the adrenal medulla as site of origin. Exp. Clin. Endocrinol. Diabetes 105, 156–162.

Horio, T., Kohno, M., Kano, H., Ikeda, M., Yasunari, K., Yokokawa, K., Minami, M., Takeda, T., 1995. Adrenomedullin as a novel antimigration factor of vascular smooth muscle cells. Circ. Res. 77, 660–664.

Jaffe, E.A., Mosher, D.F., Neiman, R.S., Dervan, P., Haudenschild, C., Jaffe, R., Weksler, B.B., Marcus, A.J., Nanba, K., Jaffe, E.S., Soban, E.J., Braylan, R.C., Berard, C.W., Moore, A., Becker, C.G., Nachman, R.L., Minick, C.R., Adelman, B., Nachman, R., Gibofsky, A., Fotino, M., Hoyer, L.W., 1978. Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria. Ann. N. Y. Acad. Sci. 312, 122–131.

Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuo, H., Eto, T., 1993. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem. Biophys. Res. Commun. 192, 553–560.

Radomski, M.W., Palmer, R.M., Moncada, S., 1987. The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. Br. J. Pharmacol. 92, 639–646.

Ribeiro, S.M., Schultz-Cherry, S., Murphy-Ullrich, J.E., 1995. Heparin-binding vitronectin up-regulates latent TGF-beta production by bovine aortic endothelial cells. J. Cell Sci. 108, 1553–1561.

Schiller, N.K., Champion, H.C., Hugghins, S.Y., Timothy, A.M., Murphy, W.A., Coy, D.H., Peter, J.R., Kadowitz, P.J., McNamara, D.B., 1998. Adrenomedullin does not inhibit human platelet aggregation. J. Cardiovasc. Pharmacol. Ther. 3, 223–228.

Shimekake, Y., Nagata, K., Ohta, S., Kambayashi, Y., Teraoka, H., Kitamura, K., Eto, T., Kangawa, K., Matsuo, H., 1995. Adrenomedullin stimulates two signal transduction pathways, cAMP accumulation and $Ca2+$ mobilization, in bovine aortic endothelial cells. J. Biol. Chem. 270, 4412–4417.

Sugo, S., Minamino, N., Kangawa, K., Miyamoto, K., Kitamura, K., Sakata, J., Eto, T., Matsuo, H., 1994. Endothelial cells actively synthesize and secrete adrenomedullin. Biochem. Biophys. Res. Commun. 201, 1160–1166.