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Biochemical and Biophysical Research Communications 325 (2004) 80–84
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Adrenomedullin induces matrix metalloproteinase-2 activity in rat aortic adventitial fibroblasts

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Received 27 September 2004
Available online 22 October 2004

Abstract

Background. The delicate balance of the extracellular matrix (ECM) determines the stiffness of the vascular wall, and adventitial fibroblasts are involved in ECM formation by synthesizing and degrading matrix proteins. In the present study, we examined the effect of the bioactive peptide adrenomedullin (AM) on activity and expression of matrix metalloproteinases (MMPs) in cultured aortic adventitial fibroblasts.
Methods and results. In cultured adventitial fibroblasts isolated from aorta of adult Wistar rats, 10^{-6} mol/L angiotensin II (Ang II) significantly ($p < 0.05$) down-regulated MMP-2 activity as determined by in vitro gelatin zymography. In contrast, 10^{-7} mol/L synthetic rat AM significantly ($p < 0.05$) stimulated zymographic MMP-2 activity by 23%, increasing intracellular cAMP, and AM abolished the action of Ang II, augmenting the MMP-2 activity. Similarly, Ang II down-regulated MMP-2 protein expression assessed by Western blotting, whereas AM increased it. Furthermore, 8-bromo-cAMP, an analogue of cAMP, mimicked the effect of AM, and H-89, an inhibitor for protein kinase A (PKA), significantly decreased the basal and AM-induced MMP-2 activity.
Conclusion. This study provides a new insight into the biological action of AM and its intracellular signaling system of cAMP/PKA stimulating the matrix degrading enzyme MMP-2, suggesting an important role for this molecule in modulating ECM deposition in the adventitial layer.

Keywords: Adrenomedullin; Vascular remodeling; Collagen; Adventitia; Matrix metalloproteinase

Vascular remodeling, defined as a change of vessel size, is initially a physiological adaptation to physical stimuli, such as blood flow, shear stress, and wall tension, to preserve the luminal size of the vessels [1,2]. However, the remodeling process becomes maladaptative in diseased situations such as atherosclerosis or vascular injury, leading to inappropriate extracellular matrix (ECM) reorganization. The delicate balance of ECM synthesis and degradation determines the level of ECM deposition in the vascular wall. A group of Ca^{2+}- and Zn^{2+}-dependent endopeptidases, matrix metalloproteinases (MMPs), contribute to the vascular remodeling by degrading several ECM proteins [3]. MMPs are present in the vasculature: MMPs-1, -14 in endothelial cells, MMPs-1, 2, 3, 9, 14 in smooth muscle cells [3], and MMPs-2, -9 in adventitial fibroblasts [4]. The adventitial layer of vascular wall has recently been recognized to have a significant role in the process of vascular remodeling [5], and either the systemic or local

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.10.014

renin–angiotensin system plays a pivotal role in vascular fibrosis by stimulating proliferation and collagen production of adventitial fibroblasts [6,7].

Enhanced ECM deposition makes the vascular wall stiffer, subsequently increasing peripheral resistance [2], therefore pharmacological intervention to ameliorate ECM deposition might be attractive in maintaining a distensibility of the vasculature. The bioactive peptide adrenomedullin (AM), initially isolated from human pheochromocytoma [8], has been shown to be produced by the cardiovascular tissues where it exerts multiple actions mainly via the intracellular cyclic AMP (cAMP) [9]. For example, AM has been found to inhibit cellular proliferation and collagen synthesis of cardiac fibroblasts in vitro [10,11]. Based upon the action of AM on the fibroblasts in the heart, we hypothesized that AM plays an important role in the vascular remodeling by affecting the ECM turnover. In the present study, we conducted in vitro experiments to examine whether AM affects expression and activity of the matrix degrading enzyme MMPs in cultured fibroblasts isolated from rat aortic adventitia.

Methods

The present study was performed in accordance with the Animal Welfare Act and with approval of the University of Miyazaki Institutional Animal Care and Use Committee (2003-023).

Materials. Dulbecco's modified Eagle's medium (DMEM)/F12 and fetal bovine serum were from Gibco-BRL. Synthetic rat AM was purchased from Peptide Institute (Osaka, Japan), H-89 was from Seikagaku (Tokyo, Japan), and other reagents were from Sigma or otherwise indicated in the text.

Cell culture. Adventitial fibroblasts of aorta were prepared according to the method of Gu and Brecher with our modification [12]. Aortic tissues resected aseptically from 10-week-old male Wistar rats were placed in ice-cold DMEM/F-12 containing 10% fetal bovine serum and antibiotics. Loosely adhering connective tissue was rapidly removed from the aorta, and the luminal surface was opened by a longitudinal cut. Endothelial cells were removed by gently rubbing the lumen with the blunt side of dissecting scissors, and the medial layer was peeled off with the use of two forceps. The remaining tissues, predominantly adventitia, were cut into segments ~2 mm^3 and placed in DMEM/F-12 solution for subsequent enzymatic digestion with 0.12% trypsin and 0.03% collagenase. The cells were then incubated in 10 cm collagen type 1-coated culture plates. After achieving confluence, they were harvested with trypsin and used for experiments at passage 3–5.

The cultured cells were incubated in DMEM/F12 medium with 10% fetal bovine serum, followed by the incubation with serum-free medium containing 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite for 24 h. Thereafter, the cells were incubated with fresh serum-free medium described above in the presence or absence of angiotensin II (Ang II), synthetic rat AM or 8-bromo-cAMP (8-Br-cAMP). In another set of experiments, H-89, a specific protein kinase A (PKA) inhibitor, was added 30 min prior to AM administration. The isolated cells were positive for vimentin, α-smooth muscle actin, but negative for von-Willebrand factor, desmin, smooth muscle-myosin heavy chain and caldesmon, indicating that they are activated adventitial fibroblasts.

In vitro gelatin zymography. Conditioned media of adventitial fibroblasts (2 μg protein) were concentrated and used for the zymographic gelatinase activity assay as described before [13]. In brief, samples mixed with Laemmli sample buffer were loaded onto 10% Tris–glycine gels with 0.1% gelatin (Invitrogen) in Tris–glycine sodium dodecyl sulfate running buffer. The gels were then washed with renaturing buffer, followed by incubation with zymogen developing buffer for 24 h. They were stained with 0.5% (wt/vol) Coomassie brilliant blue overnight and then with destaining buffer. Lytic bands corresponding to the pro- and active-forms of MMP-2 were analyzed as a total MMP-2 activity with NIH image software v. 1.63.

Western blot. Denatured protein extracts (30 or 60 μg) from cultured adventitial fibroblasts were subjected to sodium dodecyl sulfate–polyacrylamide gel as previously described [13]. The separated proteins were electrically transferred onto polyvinylidene difluoride membranes (Bio-Rad). Equal protein loading was verified by staining the gels with Coomassie brilliant blue. After blocking the non-specific background with 5% skim milk, polyvinylidene difluoride membranes were incubated with anti-MMP-2 polyclonal antibody (AB809, Chemicon, 1:1000), anti-tissue inhibitor of MMP-2 (TIMP-2) polyclonal antibody (AB801, Chemicon, 1:1000) or anti-MMP-14 monoclonal antibody (Daiichi Fine Chemical F-84, 10 μg/mL) followed by incubation with horseradish peroxidase-coupled secondary antibody. Immunoreactive bands were visualized by the ECL Plus detection kit (Amersham), and the intensity of the bands was analyzed densitometrically (Chemi Doc Documentation System, Bio-Rad).

Assays for cAMP and AM. Intracellular cAMP concentration in the adventitial fibroblasts was measured as previously described [10]. In brief, the fibroblasts were incubated in Hanks' balanced salt solution containing 20 mmol/L *N*-[2-hydroxyethyl]piperazine-3-isobutyl-1-methylxanthine for 10 min at 37 °C. AM was then added at the indicated concentrations. The reaction was terminated by aspirating the medium and adding cold 6% trichloroacetic acid. Cells were extracted with water-saturated ethyl ether, and cAMP was measured by radioimmunoassay. To evaluate AM secretion from the adventitial fibroblasts, conditioned media were collected at indicated time points, and rat AM immunoreactivity in the media was measured with commercially available immunoradiometric assay kits (Shionogi, Japan).

Statistical analysis. All data are expressed as means ± SEM. Results of the Western blot and zymography are expressed as ratios relative to the respective control. Comparisons between groups were assessed with one-way ANOVA followed by Fisher's test. A statistical significance was accepted at $p < 0.05$.

Results

Effects of Ang II and AM on MMP-2 activity and protein

Fig. 1A illustrates the effects of Ang II and AM on zymographic MMP-2 activity in the cultured aortic adventitial fibroblasts. One μmol/L of Ang II significantly ($p < 0.05$) decreased zymographic MMP-2 activity in these cells. Conversely, 10^{-7} mol/L synthetic rat AM significantly increased MMP-2 activity by 23% ($p < 0.05$), and AM abolished the action of Ang II, augmenting MMP-2 activity, at 10^{-7} and 10^{-8} mol/L. MMP-9 is another metalloproteinase having an important role in the adventitia [4], but the band corresponding to MMP-9 activity of 92 kDa was too faint to be quantified in control fibroblasts and those stimulated by either Ang II or AM. Fig. 1B illustrates the effects of Ang II and AM on MMP-2 protein expression. Similar to the zymographic finding, 10^{-6} mol/L Ang II down-regulated protein expression of MMP-2, while 10^{-7} mol/L AM increased the MMP-2 expression whether or not Ang II was added.

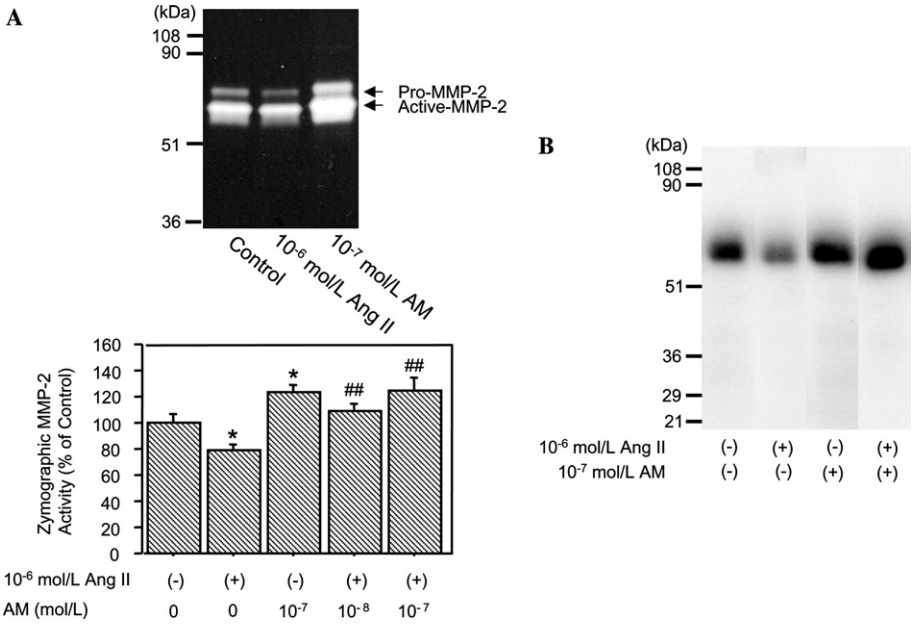

Fig. 1. (A) Effects of Ang II and AM on zymographic MMP-2 activity in cultured adventitial fibroblasts. Adventitial fibroblasts were incubated with or without 10^{-6} mol/L Ang II in the absence or presence of indicated concentration of synthetic rat AM for 24 h. Values are shown as means ± SEM ($n = 7$–8). The upper panel shows a representative zymographic picture. $^{*}p < 0.05$, vs. control cells; $^{\#\#}p < 0.01$, vs. cells incubated with Ang II. (B) Effects of Ang II and AM on protein expression of MMP-2 in adventitial fibroblasts. Identical results were obtained in three independent experiments.

Roles of cAMP/PKA signaling on zymographic MMP-2 activity

Figs. 2A and B illustrate the effect of AM on intracellular cAMP content in cultured adventitial fibroblasts. One μmol/L AM significantly stimulated cAMP production, peaking at 10 min (Fig. 2A), and this effect was dose-dependent (Fig. 2B). Figs. 2C and D illustrate the effects of the cAMP analogue 8-Br-cAMP and the specific PKA inhibitor H-89 on zymographic MMP-2 activity. 8-Br-cAMP mimicked the effect of AM stimulating MMP-2 activity (Fig. 2C), while H-89 significantly decreased the MMP-2 activity of the cells incubated with or without 10^{-7} mol/L AM (Fig. 2D).

AM secretion from adventitial fibroblasts

Adventitial fibroblasts time-dependently secreted AM into the media up to 48 h under the serum-free conditions. Rat AM concentrations in the conditioned media were: 6 h, 1.4 ± 0.1; 24 h, 2.0 ± 0.3; and 48 h, 5.5 ± 0.3 fmol/10^5 cells ($n = 6$).

Discussion

Balance between ECM synthesis and degradation determines its abundance in the vasculature. Although hemodynamic stimuli largely regulate vascular remodeling in normal arteries, humoral factors such as Ang II, inappropriately stimulating collagen synthesis, seem to be of importance in the pathological situation [1]. MMPs reorganize the vessel structure by degrading the ECM proteins and a significant role for MMPs in vascular remodeling has been suggested [3]. For example, the plasma level of MMP-1 was reduced in hypertensive patients [14], and MMPs-1, 3, and 9 were up-regulated in the atherosclerotic lesion [15,16]. Constitutively produced by vascular endothelial and smooth muscle cells, MMP-2 degrades gelatin, type I, IV, V, VII collagen, and elastin [17,18]. Here we report for the first time that the bioactive peptide AM increased protein level and enzymatic activity of MMP-2 in cultured aortic adventitial fibroblasts of rats. Hernandez-Barrantes et al. [19] showed that MMP-2 is regulated by MMP-14, a membranous type of MMP, and by the tissue inhibitor of MMP (TIMP)-2. We examined expressions of these proteins by Western blotting, but neither AM nor Ang II affected the MMP-14 protein expression and TIMP-2 was undetectable in the fibroblasts (data not shown).

Renin–angiotensin system activation has been shown to be involved in the process of vascular remodeling. Angiotensinogen is expressed in the adventitia [20], and angiotensin converting enzyme was found to be induced in the injured artery [21]. Further, mast cell mainly distributed in the adventitia has been reported to be an additional source of renin [22]. These findings suggest an existence of the local renin–angiotensin system in vascular adventitia. Recent reports have shown that MMP-2 activity is down-regulated in the

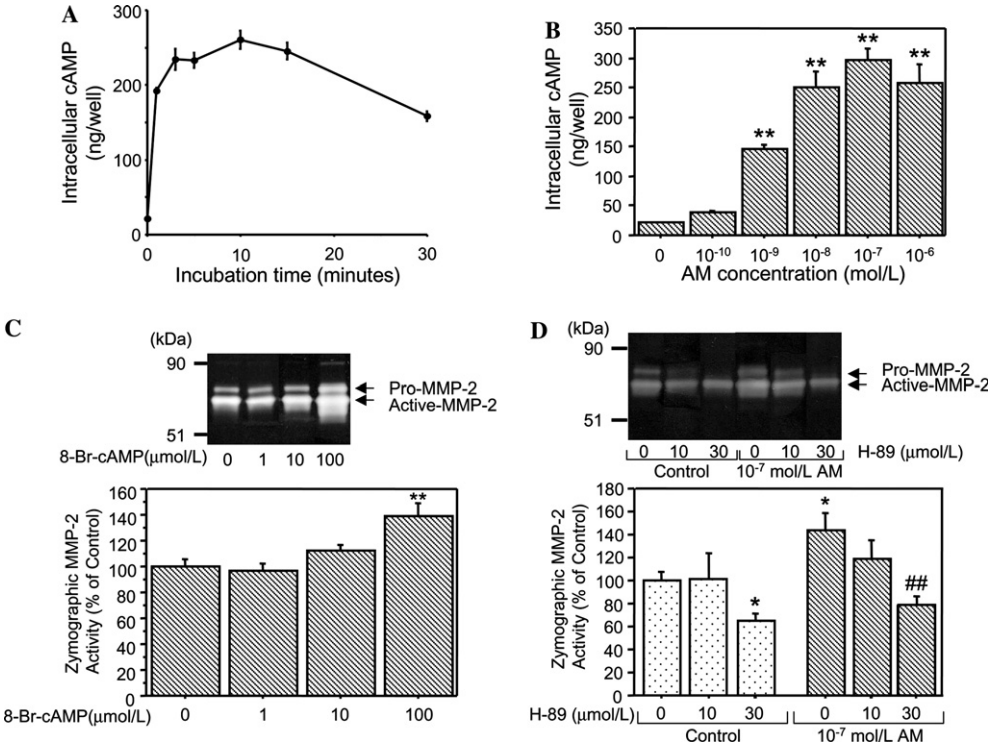

Fig. 2. (A,B) Time course of cAMP elevation by 10^{-6} mol/L rat synthetic AM (A) and dose-dependent effect of AM on cAMP elevation at 10 min (B) in adventitial fibroblasts. (C,D) Effects of 8-Br-cAMP (C) and H-89 (D) on zymographic MMP-2 activity in adventitial fibroblasts. The upper panels show representative zymographic pictures. Values are shown as means ± SEM, and the sample numbers were four in (A) and (B) and six in (C) and (D), respectively. $^{*}p < 0.05$, $^{**}p < 0.01$, vs. control fibroblasts; $^{\#\#}p < 0.01$, vs. AM-treated cells without H-89.

vasculature of rats and humans with diabetes mellitus [23,24], where the renin–angiotensin system has been shown to be activated; though, the role of AM in the vascular adventitia remains unknown. In the present study, we examined the effects of Ang II and AM on the ECM metabolism in the cultured adventitial fibroblasts. When assessed by [^{3}H]proline incorporation into the cells, AM failed to inhibit the Ang II-induced de novo collagen synthesis (data not shown). However, AM increased not only basal levels of MMP-2 protein expression and its enzymatic activity but also those reduced by Ang II.

AM was initially isolated during experiments monitoring cAMP elevation in rat platelets [8], and many of the actions of AM have been shown to be mediated by intracellular cAMP [9–11]. In the present study, the cAMP analogue 8-Br-cAMP mimicked the effect of AM, stimulating zymographic MMP-2 activity, and PKA inhibition with H-89 attenuated its activity. These results suggest that the cAMP/PKA signaling system is involved in the AM-induced activation and up-regulation of MMP-2 in the aortic adventitial fibroblasts. This is comparable with the finding by Maioli et al. [25] who reported that parathyroid hormone-related peptide stimulated MMP-2 activity by intracellular cAMP accumulation without affecting collagen synthesis in human skin fibroblasts.

It has been well documented that AM is produced by the vascular wall, particularly in endothelial and smooth muscle cells [26]. Immunohistochemical staining for AM was also observed in the adventitia of rat femoral artery [27], and consistent with this, we found that cultured aortic adventitial fibroblasts also secreted AM in this study. According to our unpublished observation, intravenous AM infusion attenuated collagen accumulation in the adventitia following ballooning injury in rat carotid artery, suggesting an important role of AM as an anti-fibrotic factor in the vascular remodeling. Taken together, renin–angiotensin system activation increases ECM formation by stimulating collagen synthesis and by decreasing MMP-2 activity in the adventitia, making the vascular wall stiffer; while AM may reduce vascular stiffness by enhancing action of the matrix degradation enzyme MMP-2, thus antagonizing the action of Ang II in the adventitial layer. However, this hypothesis should be tested further by in vivo studies.

In summary, this study provides a new insight into the biological action of AM in vascular remodeling, suggesting that AM may modulate ECM metabolism by augmenting the MMP-2 action in the adventitia.

Acknowledgments

This study was supported by the Grants-in-Aid for Scientific Research and for the 21st Century Centers of

Excellence Program (Life Science) from the Ministry of Education, Culture, Sport, Science and Technology, Japan, and by a Grant-in-Aid from AstraZeneca Research 2003.

References

[1] M.R. Ward, G. Pasterkamp, A.C. Yeung, C. Borst, Arterial remodeling. Mechanisms and clinical implications, Circulation 102 (2000) 1186–1191.
[2] H.D. Intengan, E.L. Schiffrin, Vascular remodeling in hypertension: roles of apoptosis, inflammation, and fibrosis, Hypertension 38 (2001) 581–587.
[3] Z.S. Galis, J.J. Khatri, Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly, Circ. Res. 90 (2002) 251–262.
[4] W.J. Cai, S. Koltai, E. Kocsis, D. Scholz, S. Kostin, X. Luo, W. Schaper, J. Schaper, Remodeling of the adventitia during coronary arteriogenesis, Am. J. Physiol. Heart Circ. Physiol. 284 (2003) H31–H40.
[5] S. Sartore, A. Chiavegato, E. Faggin, R. Franch, M. Puato, S. Ausoni, P. Pauletto, Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant, Circ. Res. 89 (2001) 1111–1121.
[6] K.T. Weber, Y. Sun, E. Guarda, Structural remodeling in hypertensive heart disease and the role of hormones, Hypertension 23 (1994) 869–877.
[7] D.K. Kim, J.E. Huh, S.H. Lee, K.P. Hong, J.E. Park, J.D. Seo, R.W. Lee, Angiotensin II stimulates proliferation of adventitial fibroblasts cultured from rat aortic explants, J. Korean Med. Sci. 14 (1999) 487–496.
[8] K. Kitamura, K. Kangawa, M. Kawamoto, Y. Ichiki, S. Nakamura, H. Matsuo, T. Eto, Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma, Biochem. Biophys. Res. Commun. 192 (1993) 553–560.
[9] K. Kitamura, K. Kangawa, T. Eto, Adrenomedullin and PAMP: discovery, structures, and cardiovascular functions, Microsc. Res. Tech. 57 (2002) 3–13.
[10] T. Tsuruda, J. Kato, K. Kitamura, M. Kawamoto, K. Kuwasako, T. Imamura, Y. Koiwaya, T. Tsuji, K. Kangawa, T. Eto, An autocrine or a paracrine role of adrenomedullin in modulating cardiac fibroblast growth, Cardiovasc. Res. 43 (1999) 958–967.
[11] T. Horio, T. Nishikimi, F. Yoshihara, H. Matsuo, S. Takishita, K. Kangawa, Effects of adrenomedullin on cultured rat cardiac myocytes and fibroblasts, Eur. J. Pharmacol. 382 (1999) 1–9.
[12] M. Gu, P. Brecher, Nitric oxide-induced increase in p21 (Sdi1/Cip1/Waf1) expression during the cell cycle in aortic adventitial fibroblasts, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 27–34.
[13] T. Tsuruda, G. Boerrigter, B.K. Huntley, J.A. Noser, A. Cataliotti, L.C. Costello-Boerrigter, H.H. Chen, J.C. Burnett Jr., Brain natriuretic peptide is produced in cardiac fibroblasts and induces matrix metalloproteinases, Circ. Res. 91 (2002) 1127–1134.
[14] C. Laviades, N. Varo, J. Fernández, G. Mayor, M.J. Gil, I. Monreal, J. Díez, Abnormalities of the extracellular degradation of collagen type I in essential hypertension, Circulation 98 (1998) 535–540.
[15] Z.S. Galis, G.K. Sukhova, M.W. Lark, P. Libby, Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques, J. Clin. Invest. 94 (1994) 2493–2503.
[16] S.T. Nikkari, K.D. O'Brien, M. Ferguson, T. Hatsukami, H.G. Welgus, C.E. Alpers, A.W. Clowes, Interstitial collagenase (MMP-1) expression in human carotid atherosclerosis, Circulation 92 (1995) 1393–1398.
[17] F.G. Spinale, Matrix metalloproteinases: regulation and dysregulation in the failing heart, Circ. Res. 90 (2002) 520–530.
[18] R.M. Senior, G.L. Griffin, C.J. Fliszar, S.D. Shapiro, G.I. Goldberg, H.G. Welgus, Human 92- and 72-kilodalton type IV collagenases are elastases, J. Biol. Chem. 266 (1991) 7870–7875.
[19] S. Hernandez-Barrantes, M. Toth, M.M. Bernardo, M. Yurkova, D.C. Gervasi, Y. Raz, Q.A. Sang, R. Fridman, Binding of active (57 kDa) membrane type 1-matrix metalloproteinase (MT1-MMP) to tissue inhibitor of metalloproteinase (TIMP)-2 regulates MT1-MMP processing and pro-MMP-2 activation, J. Biol. Chem. 275 (2000) 12080–12089.
[20] L.A. Cassis, K.R. Lynch, M.J. Peach, Localization of angiotensinogen messenger RNA in rat aorta, Circ. Res. 62 (1988) 1259–1262.
[21] J.L. Zhuo, P. Froomes, D. Casley, J.J. Liu, C. Murone, Y.S. Chai, B. Buxton, F.A. Mendelsohn, Perindopril chronically inhibits angiotensin-converting enzyme in both the endothelium and adventitia of the internal mammary artery in patients with ischemic heart disease, Circulation 96 (1997) 174–182.
[22] R.B. Silver, A.C. Reid, C.J. Mackins, T. Askwith, U. Schaefer, D. Herzlinger, R. Levi, Mast cells: a unique source of renin, Proc. Natl. Acad. Sci. USA 101 (2004) 13607–13612.
[23] S. Jesmin, I. Sakuma, Y. Hattori, A. Kitabatake, Role of angiotensin II in altered expression of molecules responsible for coronary matrix remodeling in insulin-resistant diabetic rats, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 2021–2026.
[24] V. Portik-Dobos, M.P. Anstadt, J. Hutchinson, M. Bannan, A. Ergul, Evidence for a matrix metalloproteinase induction/activation system in arterial vasculature and decreased synthesis and activity in diabetes, Diabetes 51 (2002) 3063–3068.
[25] E. Maioli, V. Fortino, C. Torricelli, B. Arezzini, C. Gardi, Effect of parathyroid hormone-related protein on fibroblast proliferation and collagen metabolism in human skin, Exp. Dermatol. 11 (2002) 302–310.
[26] T. Eto, J. Kato, K. Kitamura, Regulation of production and secretion of adrenomedullin in the cardiovascular system, Regul. Pept. 112 (2003) 61–69.
[27] M. Yamasaki, J. Kawai, T. Nakaoka, T. Ogita, A. Tojo, T. Fujita, Adrenomedullin overexpression to inhibit cuff-induced arterial intimal formation, Hypertension 41 (2003) 302–307.