the diagnostic criteria for dementia (diagnostic and statistical manual of mental disorders; DSM-IV) [1] in the occasion of diagnosis and, most of these patients suffered in a bed-ridden condition in their terminal stages.

The diagnosis of AD was made based on the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) diagnostic neuropathologic criteria [15] and excluded the brains with large cerebral infarctions. The diagnosis of BD was made clinico-pathologically, and retrospectively met the pathological inclusion criteria including (1) presence of diffuse white matter lesions, (2) lacunar infarctions in the perforator territory, (3) arteriolosclerosis such as fibrohyalinosis and fibrinoid necrosis and (4) absence of cortical infarctions, as well as the clinical criteria by Bennett et al. [3], and excluded the brains with significant pathologic hallmarks of AD. The control group included two patients with pneumonia, one patient each with renal cancer, lung cancer, chronic renal failure and pulmonary emphysema.

The tissue blocks were sectioned in a cryostat (30 or 100 μm-thick) and kept in 0.1 M phosphate buffer (pH 7.4). The tissue sections were processed according to the silver impregnation method described by Gallyas [8]. Briefly, the sections were incubated for 30 min in 5% periodic acid (HIO4) solution. The sections were then immersed in 4% sodium hydroxide solution for 30 min, and washed in 0.5% acetic acid solution in double-distilled water for 5 min. They were further incubated in a mixture (pH 13.0) of 90 ml of an ammoniated silver nitrate solution (AgNO3, 0.5 g and ammonium nitrate, 2.5 g in 900 ml double-distilled water), and 10 ml of 4% sodium dihydroxide in double-distilled water for 30 min at 20 °C. These sections were left in a physical developer solution, which was composed of a mixture of 10 ml of solution A, 5 ml of solution B and 5 ml of solution C at 25 °C. The composition of each solution was as follows; sodium carbonate, 50 g in 1000 ml of distilled water (solution A); ammonium nitrate 1.9 g, silver nitrate, 2.0 g; tungsto-silicic acid ($SiO_2 \cdot 2WO_3$), 10 g in 1000 ml of distilled water (solution B); and ammonium nitrate, 1.9 g; silver nitrate, 2.0 g; tungsto-silicic acid ($SiO_2 \cdot 2WO_3$), 10.0 g; and 6.1 ml of 40% formalin solution in 1000 ml of distilled water (solution C). The reaction was terminated in 0.5% acetic acid solution, and the extent of silver impregnation was monitored intermittently under light microscopy.

For immunohistochemistry, autoclaved paraffin sections were incubated with a mouse anti-amyloid β protein antibody (Dakopatts, diluted 1:200), biotinylated anti mouse IgG (Vector laboratories, diluted 1:200) and an avidin biotinylated peroxidase complex (Vector Laboratories, diluted 1:200). They were finally visualized with 0.01% diaminobenzidine tetrahydrochloride and 0.005% H_2O_2 in 0.05 M Tris–HCl (pH 7.6). To test for the specificity of the immunohistochemical reaction, control sections were incubated with normal mouse IgG instead of the primary antibody.

The density of the capillary beds was determined by the test grid method [7], in which the number of vascular intersections were counted against 6 × 6 square test grids each with a 50 μm width. The average counts from five representative fields in the layers II–IV of the frontal and parietal cortices, respectively, were used as the capillary densities in each patient. The data were expressed as means ± S.D. and the Mann–Whitney *U*-test was used to compare between the groups.

Using the modified Gallyas stains, the microvessels in the cerebral cortices appeared smooth and regular in diameter in the non-neurological control and BD brains (Fig. 1A and C). There were no senile plaques nor neurofibrillary tangles. In contrast, the brains with AD had numerous senile plaques and neurofibrillary tangles, which were intermingled by irregularly-shaped microvessels in both frontal and parietal cortices (Fig. 1B). The microvessels were frequently narrowed and irregular in diameter for a variable length of the vessel (Fig. 1D–F). These vessels often showed bulging of their walls. In close proximity to the senile plaques, the microvessels were blunted and torn off in the sections with a thickness of 100 μm (Fig. 1E). These microscopic changes were not observed in the non-neurological control and BD groups.

With immunohistochemistry, β amyloid-immunoreactivity was localized in senile plaques which accumulated numerously in the superficial layer, as well as perivascular deposits in the vascular wall itself and perivascular neuropil in the AD group (Fig. 1G–I). Beta amyloid-immunopositive fine texture fibrils were distributed in the neuropil with or without contact to the microvessels. In contrast, there was almost no deposit of β amyloid in the cerebral cortices of the non-neurological control and BD groups. In the semi-quantitative measures of the microvessels, the microvascular densities were significantly lower in the AD group as compared to the other two groups in both frontal and parietal cortices (Fig. 2).

The capillaries in AD have been shown to exhibit thickening of their basement membrane, atrophy, perivascular fibrosis and degeneration of the pericytes [5,6,16], which may correspond to the bulging of the microvessels observed here. In semi-quantitative measures, some authors have not observed any decrease in the capillary densities [2], while others showed a decrease in selected or non-selected regions of AD brains [4,7]. The present study underscored the morphological abnormalities of the capillaries, and further revealed their numerical decrease in AD and absence of capillary damages in BD. The actual reduction rate in the capillary density of AD brains may be more severe, because significant atrophy in this group should have ameliorated the reduction ratio. The fact that there were no β amyloid-deposits nor damages in the cortical microvessels in BD brains was not contradictory to the major site of the pathologic process, which involve subcortical white matter and perforator territory in BD. However, in previous studies, slight but significant neuronal dysfunction has been noted in the cerebral cortex, such as a decrease in the synaptic densities and neuronal viabilities [11,22].

The reduction in the vascular densities and the spatial proximity of β amyloid deposits to the microvascular changes may suggest some vascular toxicity due to β amyloid. Indeed, preamyloid deposits were found in the extracellular space and extended directly into the capillaries [16]. Vinters and Farag [20] raised a neurovascular hypothesis, in which β amyolid accumulates on the outer side of the basement membrane and

Please cite this article in press as: H. Kitaguchi et al., Capillary beds are decreased in Alzheimer's disease, but not in Binswanger's disease, Neurosci. Lett. (2007), doi:10.1016/j.neulet.2007.02.021

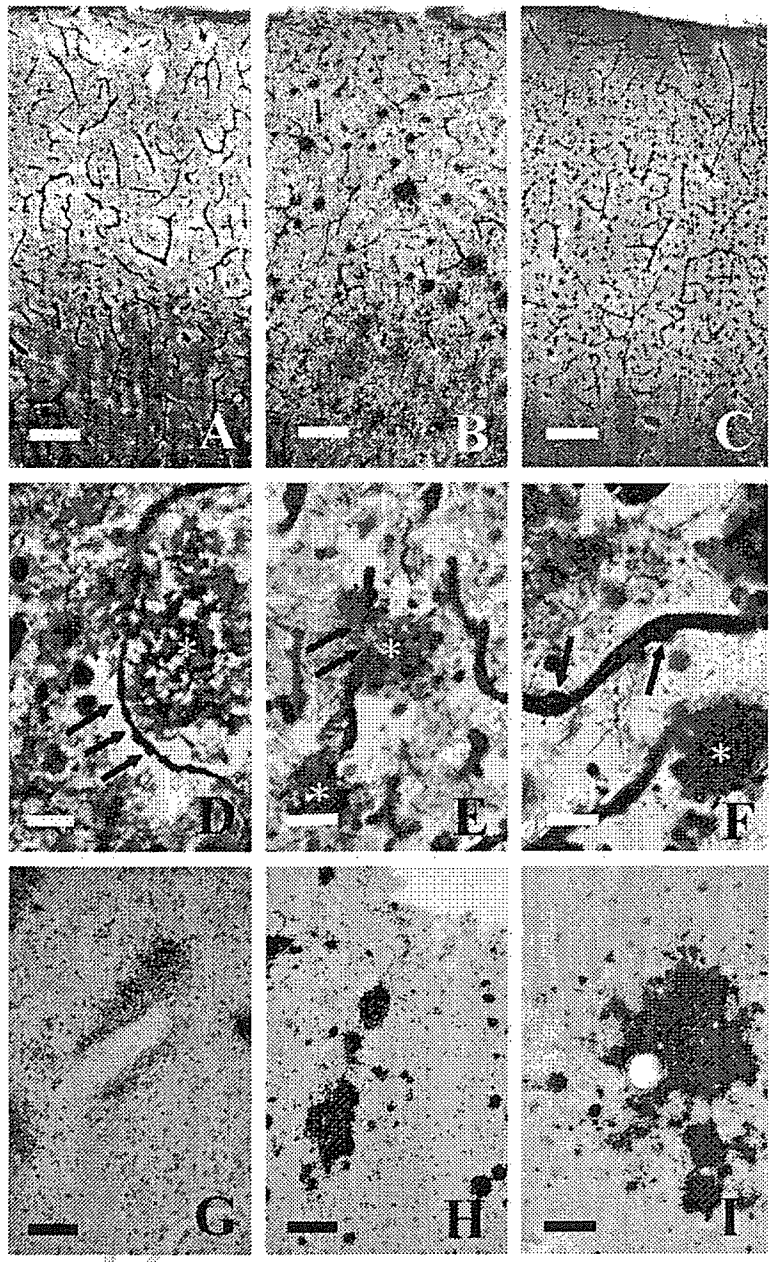

Fig. 1. Photomicrograph of Gallyas stains (A)–(F) and immunohistochemistry for β amyloid (G)–(I). The photographs were taken in the cerebral cortex of non-neurological (A); AD (B), (D)–(F); and BD patients (C). Numerous senile plaques were observed exclusively in AD brains. The capillary density appears less dense in AD as compared with the non-neurological and BD brains. The small vessels showed narrowing (D), tearing off (E) and scattered bulging (F). There were perivascular deposit in the vascular wall which extended into the neuropil diffusely (G). A heavy accumulation of perivascular β amyloid was also seen in the tangential (H) and axial planes (I), in which the perivascular deposits continued into the neuroglial deposits. Bars indicate 100 μm for (A)–(C), (I), 30 μm for (D)–(F) and 200 μm in (G), (H).

may promote local neurovascular inflammation. In support for this hypothesis, GAX, a gene encoding a homeodomain-transcription factor box gene related to vascular differentiation [9], is downregulated in AD brains. The downregulation of GAX activates a proapoptotic pathway, and may result in a decrease in the number of cerebral microvessels and cerebral blood flow (CBF), by way of activating the forkhead transcription factor, AFX-1. This activation may also downregulate low density lipoprotein receptor-related prtoein-1 (LRP) which enhances efflux of β amyloid from the brain [21]. This impaired clearance of β amyloid may further increase soluble β amyloid and fibrillary β amyloid levels [23]. Finally, we hypothesized a vicious cycle in which β amyloid may cause microvascular regression, brain hypoperfusion and neurovascular inflammation, although this will be addressed in future studies.

Please cite this article in press as: H. Kitaguchi et al., Capillary beds are decreased in Alzheimer's disease, but not in Binswanger's disease, Neurosci. Lett. (2007), doi:10.1016/j.neulet.2007.02.021

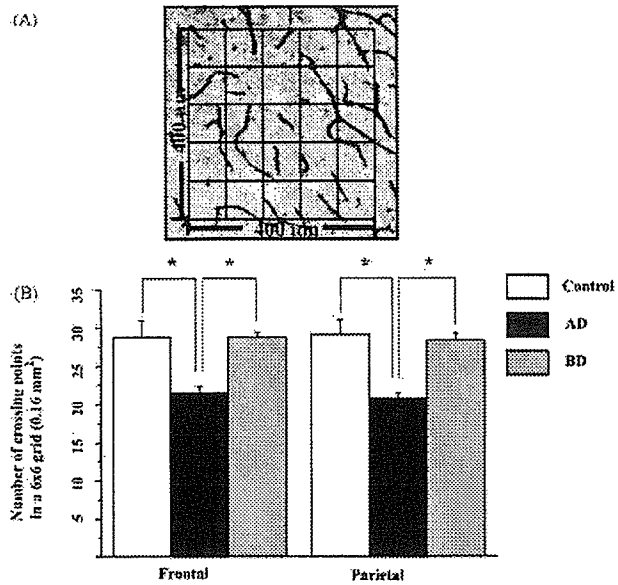

Fig. 2. Quantitative evaluation of capillary densities in the cerebral cortex. (A) indicates the grid applied for a non-neurological control brain; (B) indicates the capillary densities in the non-neurological, AD and BD brains. $*p < 0.05$ by Mann–Whitney U-test.

Acknowledgement

This study was supported in part by a grant-in-aid for scientific research (C) (18590936) from the Japanese Ministry of Education, Culture, Sports, Science and Technology to H. S. and H. T.

References

[1] American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders, fourth ed., Washington, DC, American Psychiatric Association, 1994.
[2] M.A. Bell, M.J. Ball, Morphometric comparison of hippocampal microvasculature in ageing and demented people: diameters and densities, Acta Neuropathol. 53 (1981) 299–318.
[3] D.A. Bennett, R.S. Wilson, D.W. Gilley, J.H. Fox, Clinical diagnosis of Binswanger's disease, J. Neurol. Neurosurg. Psychiatry 53 (1990) 961–965.
[4] L. Buee, P.R. Hof, C. Bouras, A. Delacourte, D.P. Perl, J.H. Morrison, H.M. Fillit, Pathological alterations of the cerebral microvasculature in Alzheimer's disease and related dementing disorders, Acta Neuropathol. 87 (1994) 469–480.
[5] J.C. de la Torre, Hemodynamic consequences of deformed microvessels in the brain in Alzheimer's disease, Ann. N Y Acad. Sci. 826 (1997) 75–91.
[6] E. Farkas, P.G. Luiten, Cerebral microvascular pathology in aging and Alzheimer's disease, Prog. Neurobiol. 64 (2001) 575–611.
[7] V.W. Fischer, A. Siddiqi, Y. Yusufaly, Altered angioarchitecture in selected areas of brains with Alzheimer's disease, Acta Neuropathol. 79 (1990), pp. 672–629.
[8] F. Gallyas, Silver staining of collagen and reticulin fibers and cerebral capillaries by mean of physical development, J. Microscopy 91 (1970) 119–124.
[9] D.H. Gorski, K. Walsh, Control of vascular cell differentiation by homeobox transcription factors, Trends Cardiovasc. Med. 13 (2003) 213–220.
[10] V. Hachinski, D.G. Munoz, Cerebrovascular pathology in Alzheimer's disease: cause, effect or epiphenomenon? Ann. N Y Acad. Sci. 826 (1997) 1–6.
[11] M. Ihara, H. Tomimoto, K. Ishizu, T. Mukai, H. Yoshida, N. Sawamoto, M. Inoue, T. Doi, K. Hashikawa, J. Konishi, H. Shibasaki, H. Fukuyama, Decrease in cortical benzodiazepine receptors in symptomatic patients with leukoaraiosis: a positron emission tomography study, Stroke 35 (2004) 942–947.
[12] R.N. Kalaria, Similarities between Alzheimer's disease and vascular dementia, J. Neurol. Sci. 203–204 (2002) 29–34.
[13] J.-Xi. Lin, H. Tomimoto, I. Akiguchi, M. Matsuo, H. Wakita, H. Shibasaki, H. Budka, Vascular cell components of the medullary arteries in Binswanger's disease brains: a morphometric and immunoelectron microscopic study, Stroke 31 (2000) 1838–1842.
[14] G.L. Mancardi, F. Perdelli, C. Rivano, A. Leonardi, O. Bugiani, Thickening of the basement membrane of cortical capillaries in Alzheimer's disease, Acta Neuropathol. 49 (1980) 79–83.
[15] S.S. Mirra, A. Heyman, D. McKeel, S.M. Sumi, B.J. Crain, L.M. Brownlee, F.S. Vogel, J.P. Hughes, G. van Belle, L. Berg, The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease, Neurology 41 (1991) 479–486.
[16] T. Miyakawa, S. Katsuragi, K. Yamashita, K. Ohuchi, Morphological study of amyloid fibrils and preamyloid deposits in the brain with Alzheimer's disease, Acta Neuropathol. 83 (1992) 340–346.
[17] A.B. Scheibel, Alterations of the cerebral capillary bed in the senile dementia of Alzheimer, Ital. J. Neurol. Sci. 8 (1987) 457–463.
[18] D.A. Snowdon, L.H. Greiner, J.A. Mortimer, K.P. Riley, P.A. Greiner, W.R. Markesbery, Brain infarction and the clinical expression of Alzheimer disease, JAMA 277 (1997) 813–817.
[19] H. Tomimoto, I. Akiguchi, H. Akiyama, K. Ikeda, H. Wakita, J.-Xi. Lin, I. Akiguchi, M. Kinoshita, H. Shibasaki, Vascular changes in white matter lesions of Alzheimer's disease, Acta Neuropathol. 97 (1999) 629–634.
[20] H.V. Vinters, E.S. Farag, Amyloidosis of cerebral arteries, Adv. Neurol. 92 (2003) 105–112.
[21] Z. Wu, H. Guo, N. Chow, J. Sallstrom, R.D. Bell, R. Deane, A.I. Brooks, S. Kanagala, A. Rubio, A. Sagare, D. Liu, F. Li, D. Armstrong, T. Gasiewicz, R. Zidovetzki, X. Song, F. Hofman, B.V. Zlokovic, Role of the MEOX2 homeobox gene in neurovascular dysfunction in Alzheimer disease, Nat. Med. 11 (2005) 959–965.
[22] S.S. Zhan, K. Beyreuther, H.P. Schmitt, Synaptophysin immunoreactivity of the cortical neuropil in vascular dementia of Binswanger type compared with the dementia of Alzheimer type and nondemented controls, Dementia 5 (1994) 79–87.
[23] B.V. Zlokovic, Neurovascular mechanisms of Alzheimer's neurodegeneration, Trends Neurosci. 28 (2005) 202–208.

Please cite this article in press as: H. Kitaguchi et al., Capillary beds are decreased in Alzheimer's disease, but not in Binswanger's disease, Neurosci. Lett. (2007), doi:10.1016/j.neulet.2007.02.021

Physiological Significance and Therapeutic Potential of Adrenomedullin in Pulmonary Hypertension

Shinsuke Murakami[1,2], Hiroshi Kimura[2], Kenji Kangawa[3] and Noritoshi Nagaya[1,*]

[1]*Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, Osaka, Japan,* [2]*Second Department of Internal Medicine, Nara Medical University, Nara, Japan,* [3]*Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan*

Abstract: Adrenomedullin (ADM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma. Its vasodilatory effect is mediated by cyclic adenosine 3',5'-monophosphate- and nitric oxide-dependent mechanisms. Earlier studies have demonstrated that ADM is secreted from various tissues, including vessels, heart, and lungs. In addition, there are specific receptors for ADM in the lungs. Plasma ADM level is elevated in proportion to the severity of pulmonary hypertension, and circulating ADM is partially metabolized in the lungs. These findings suggest that ADM plays an important role in the regulation of pulmonary vascular tone. Administration of ADM by intravenous or intratracheal delivery significantly decreased pulmonary arterial pressure and pulmonary vascular resistance in patients with pulmonary arterial hypertension. Furthermore, we have recently developed a new therapeutic strategy using ADM gene-modified endothelial progenitor cells (EPC). Intravenously administered ADM gene-modified EPC were incorporated into lung tissues and attenuated monocrotaline-induced pulmonary hypertension in rats. In addition, ADM has angiogenic and anti-apoptotic activities *via* activation of Akt and/or mitogen-activated protein kinase. These findings suggest that ADM may act not only as a vasodilator but also as a vasoprotective factor. Thus, ADM may be a promising endogenous peptide for the treatment of pulmonary hypertension.

Key Words: Adrenomedullin, pulmonary hypertension, vasodilation, cyclic adenosine 3',5'-monophosphate, nitric oxide, endothelial progenitor cell, gene therapy, angiogenesis, anti-apoptosis, Akt.

INTRODUCTION

Idiopathic pulmonary arterial hypertension (IPAH) is a rare but life-threatening disease characterized by increasing pulmonary vascular resistance and progressive pulmonary hypertension that leads to right ventricular failure and death [1]. The median survival is estimated to be 2.8 years from diagnosis [2]. Because the presence of endothelial abnormalities in the pulmonary vascular bed causes pulmonary vasoconstriction, smooth muscle cell proliferation, and *in situ* thrombosis [3], a variety of vasodilators, anti-proliferative agents, and anticoagulants have been proposed as therapeutic agents for IPAH [4-6]. Despite therapeutic medical advances including prostacyclin therapy, some patients ultimately require heart-lung or lung transplantation [7,8]. Thus, a novel therapeutic strategy is desirable for the treatment of pulmonary hypertension.

Adrenomedullin (ADM), which was originally isolated from human pheochromocytoma, is a potent and long-lasting hypotensive peptide [9]. Human ADM consists of 52 amino acids with a single intramolecular disulfide bond and an amidated tyrosine at the carboxy terminus (Fig. **1**) [9]. This peptide shares some structural homology with calcitonin gene-related peptide and amylin. The effects of ADM are mediated by two Gs-protein-coupled plasma membrane receptors: calcitonin-receptor-like receptor and receptor activity-modifying protein-2 or -3 [10]. Subsequent studies have revealed that immunoreactive ADM is distributed in plasma and a wide range of tissues including aorta, ventricles, lungs, and kidneys [11,12]. In particular, ADM is actively produced and secreted by vascular endothelial cells and vascular smooth muscle cells [13-15]. In addition to potent vasodilating effects, ADM has been reported to have multiple effects on cardiovascular and renal function, such as diuretic and natriuretic effects [16,17], and a positive inotropic effect [18,19]. Recently, ADM has received much attention as an important factor in cell growth and survival [20-25]. Thus, ADM has come to be regarded as a multifunctional peptide.

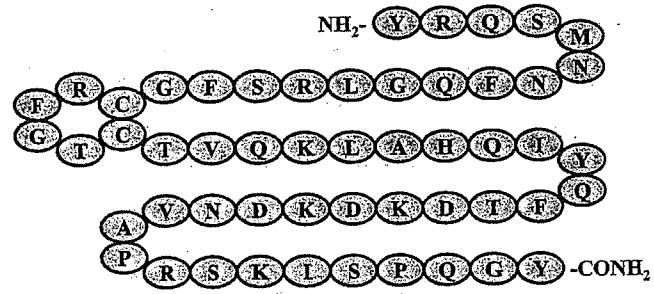

Fig. (1). Human adrenomedullin consists of 52 amino acids with a single intramolecular disulfide bond between residues 16 and 21 and with an amidated tyrosine at the carboxy terminus.

Previous studies have shown that plasma and/or tissue ADM levels are elevated in a variety of cardiovascular and renal diseases including hypertension [26-28], acute myocardial infarction [29-32], heart failure [33-39], and renal failure [40]. We and others have reported that plasma

*Address correspondence to this author at the Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan; Tel: +81-6-6833-5012; Fax: +81-6-6872-7485; E-mail: nnagaya@ri.ncvc.go.jp

1871-529X/06 $50.00+.00

ADM levels are also elevated in patients with pulmonary hypertension in proportion to the clinical severity [41-43]. These findings suggest that ADM may be involved in the regulation of cardiovascular and renal function and vascular tone. Recent studies have reported that in heterozygous ADM knockout mice with almost half the level of ADM in organs and plasma, marked cardiac hypertrophy and coronary artery lesions were observed under conditions of high cardiovascular stress [44,45]. Thus, endogenous ADM may possess a protective effect against cardiovascular damage, and increased plasma and/or tissue ADM levels may be a compensatory response to organ failure.

This review summarizes the physiological significance of ADM in patients with pulmonary hypertension and the therapeutic potential of ADM for the treatment of pulmonary hypertension.

PLASMA LEVELS OF ADRENOMEDULLIN IN PATIENTS WITH PULMONARY HYPERTENSION

Plasma ADM levels have been reported to be elevated in patients with pulmonary arterial hypertension [46] and in animal models of experimental pulmonary hypertension induced by monocrotaline [47]. To investigate the pathophysiological significance of ADM in pulmonary hypertension, we studied the relationship between plasma ADM levels and pulmonary hemodynamics in patients with pulmonary arterial hypertension [41]. Plasma ADM levels in patients with pulmonary arterial hypertension were significantly higher than those in healthy subjects. In addition, there were significant correlations between the plasma ADM level and mean pulmonary arterial pressure, total pulmonary resistance, and mean right arterial pressure. These findings suggest that the plasma ADM level is increased in proportion to the severity of pulmonary hypertension and right heart failure.

Previous studies have shown that ADM mRNA and its receptor mRNA are highly expressed in the lung [48]. Yoshibayashi *et al.* measured plasma ADM levels in blood samples obtained from various sites during cardiac catheterization in patients with pulmonary hypertension [46]. The plasma ADM level in the pulmonary vein was significantly lower than that in the pulmonary artery, consistent with the previous finding that the pulmonary circulation is the site of ADM clearance [49]. These findings suggest that elevated endogenous ADM in plasma may play an important role in the pulmonary circulation.

Previous studies have demonstrated that hypoxia, cytokine production, and shear stress induce ADM secretion by vascular cells [50-52]. An *in vitro* study has demonstrated that ADM is upregulated through a hypoxia-inducible factor-1 (HIF-1)-dependent pathway under hypoxic conditions [53]. Thus, hypoxia/HIF-1 is one of the most potent regulators of ADM production in pulmonary arterial hypertension.

BIOLOGICAL ACTIONS OF ADRENOMEDULLIN

Intravenous infusion of ADM results in potent and sustained hypotension [17,44,54,55]. ADM has been shown to increase the intracellular cyclic adenosine 3',5'-monophosphate (cAMP) level in vascular smooth muscle cells *via* its specific receptor [56,57]. The increase in cAMP by ADM activates protein kinase A (PKA), resulting in a decrease in calcium content in smooth muscle cells. On the other hand, ADM has been shown to induce vasorelaxation in a nitric oxide (NO)-dependent manner [58]. ADM induces activation of endothelial NO synthase in vascular endothelial cells *via* the Ca^{2+}/calmodulin-dependent [59] and the phosphatidylinositol 3-kinase (PI3K)/Akt-dependent pathway [60]. Thus, ADM regulates vascular tone through a cAMP-dependent mechanism and/or a NO-dependent mechanism (Fig. 2). It has been reported that there are many binding sites for ADM in the lung [61] and that ADM preferentially dilates pulmonary arterial resistance vessels [62]. These findings raise the possibility that ADM may play an important role in the regulation of pulmonary vascular tone in patients with pulmonary hypertension.

An imbalance between vasodilators and vasoconstrictors has been thought to have a key role in the development of IPAH [63]. Endothelial dysfunction decreases the production of vasodilators such as prostacyclin and NO, whereas it increases that of vasoconstrictors including thromboxane and endothelin-1 [3]. Previous studies demonstrated that prostacyclin synthase [64] and endothelial nitric oxide synthase [65] expression were decreased in the lungs of patients with pulmonary hypertension. Thus, pulmonary endothelial cells may be a therapeutic target for the treatment of pulmonary hypertension. ADM regulates growth and survival of endothelial cells [22,66,67]. ADM signaling is of particular significance in endothelial biology, since the peptide protects cells from apoptosis and promotes angiogenesis at least in part through activation of PI3K/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 in endothelial cells. These findings raise the possibility that elevated plasma ADM in patients with pulmonary hypertension may exert protective effects on pulmonary endothelial cells.

Plexiform lesions, the classic pathological finding in IPAH, are present in about one in three lung biopsy specimens [68]. These lesions have been considered an abnormal growth of modified smooth muscle cells [69]. *In vitro*, ADM has been shown to inhibit serum or platelet-derived growth factor-stimulated proliferation and migration in smooth muscle cells [70-72]. *In vivo*, continuous infusion of ADM has been shown to inhibit pulmonary smooth muscle cell proliferation in monocrotaline-induced pulmonary hypertension in rats [73]. These results suggest that ADM may have an inhibitory function in pulmonary vascular remodeling.

The overproduction of reactive oxygen species results in the progression of pulmonary vascular remodeling. ADM is recognized as a potent antioxidant. An *in vitro* study showed ADM suppressed reactive oxygen species production in a dose-dependent manner *via* activation of the cAMP-protein kinase A pathway [74]. Heterozygous ADM knockout mice housed under hypoxia showed not only severe pulmonary vascular injury but also higher levels of reactive oxygen species production [75]. These findings suggest that ADM might be one of the important compensatory substances to protect against hypoxia-induced pulmonary vascular remodeling, possibly through the suppression of reactive oxygen species.

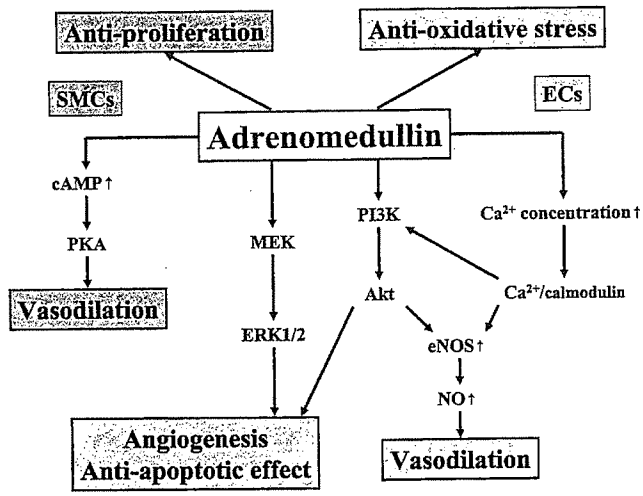

Fig. (2). Signaling pathway of adrenomedullin (ADM) in vascular endothelial cells (ECs) and smooth muscle cells (SMCs). ADM regulates vascular tone through a cAMP-dependent mechanism and/or a NO-dependent mechanism. ADM protects cells from apoptosis and promotes angiogenesis at least in part through activation of Akt and ERK1/2 in endothelial cells. ADM also inhibits smooth muscle cell proliferation and suppresses reactive oxygen species production.

THERAPEUTIC POTENTIAL OF ADRENOMEDULLIN IN PULMONARY HYPERTENSION

Previous experimental studies have shown that exogenously administered ADM causes a dose-related decrease in pulmonary arterial pressure under conditions of high pulmonary vascular tone [62,76,77]. Short-term infusion of ADM attenuates pulmonary hypertension secondary to congestive heart failure [78,79], and long-term infusion of ADM attenuates progressive pulmonary hypertension and medial thickening of the pulmonary arteries in rats treated with monocrotaline [73]. However, in humans, it remains unknown whether exogenous ADM has beneficial effects in patients with precapillary pulmonary hypertension such as IPAH or chronic thromboembolic pulmonary hypertension. Accordingly, we examined the hemodynamic and hormonal responses to intravenous infusion of ADM or placebo in 13 patients with precapillary pulmonary hypertension [80]. ADM (0.05μg/kg/minute) or placebo was randomly administered at a rate of 0.5 ml/minute for 30 minutes. At the end of ADM infusion, plasma ADM level was increased about 3-fold in the ADM group. ADM infusion produced a 44% increase in cardiac index and a 32% decrease in pulmonary vascular resistance, and its hemodynamic effects lasted at least 15 minutes after the end of infusion. These results suggest that ADM has potent, relatively long-lasting pulmonary vasodilator activity in patients with pulmonary hypertension. We have shown that plasma cAMP level was increased by 23% during ADM infusion. The increase in cAMP by ADM activates PKA, resulting in a decrease in calcium content in smooth muscle cells [57]. It is therefore possible that ADM may relax vascular smooth muscle by inducing an increase in cAMP level. ADM infusion markedly increased the cardiac index in patients with pulmonary hypertension. Considering the strong vasodilator activity of ADM in the systemic and pulmonary vasculature, a significant decrease in cardiac afterload may be responsible for the increased cardiac index with ADM. On the other hand, a previous binding study has shown abundant, specific binding sites for ADM in ventricular myocardium [61]. ADM has been shown to increase cardiac cAMP [81,82], which is known to mediate the positive inotropic action of beta-adrenergic stimulation. Alternatively, ADM has been shown to produce a positive inotropic action through cAMP-independent mechanisms [83]. These findings suggest that the increase in cardiac index may be attributable not only to a fall in cardiac afterload but also to the direct positive inotropic action of ADM. Further studies are necessary to investigate the therapeutic potential and safety of ADM in patients with pulmonary hypertension.

Intravenously administered ADM decreased systemic vascular resistance and induced systemic hypotension in patients with pulmonary hypertension because of nonselective vasodilation in the pulmonary and systemic vascular beds. Recently, inhalation of aerosolized prostacyclin and its analogue, iloprost, has been shown to cause pulmonary vasodilation without systemic hypotension in patients with IPAH [84,85]. In addition, inhalant application of vasodilators does not impair gas exchange because the ventilation-matched deposition of drug in the alveoli causes pulmonary vasodilation matched to ventilated areas. In clinical settings, inhalation therapy may be more simple, noninvasive, and comfortable than continuous intravenous infusion therapy. These findings raise the possibility that ADM inhalation may have beneficial effects in patients with precapillary pulmonary hypertension. We examined the effects of ADM inhalation on monocrotaline-induced pulmonary hypertension in rats [86]. ADM (5 μg/kg) or saline was inhaled as an aerosol using an ultrasonic nebulizer. To assess the acute effect of inhaled ADM, hemodynamic studies were carried out at 3 weeks after monocrotaline injection. Expectedly, a single 30-minute inhalation of ADM significantly decreased total pulmonary vascular resistance without a significant decrease in mean arterial pressure in rats given monocrotaline. These hemodynamic effects of ADM lasted at least 60

minutes after the end of inhalation. Furthermore, to assess the chronic effect of inhaled ADM, 30-minute inhalation of ADM or saline was repeated four times a day for 3 weeks after monocrotaline injection. Repeated inhalation of ADM markedly decreased mean pulmonary arterial pressure and total pulmonary vascular resistance without systemic hypotension in rats given monocrotaline. Interestingly, repeated inhalation of ADM significantly attenuated an increase in medial wall thickness of peripheral pulmonary arteries and improved survival. Considering the potent vasoprotective effects of ADM such as vasodilation and inhibition of smooth muscle cell migration and proliferation, it is interesting to speculate that ADM trapped in the bronchial epithelium or alveoli leaks to the pulmonary arteries to maintain pulmonary vascular integrity in rats given monocrotaline. von der Hardt *et al.* have reported that aerosolized ADM (6 μg/kg) resulted in a sustained reduction in mean pulmonary arterial pressure in a surfactant-depleted piglet model [87]. There was no significant difference in mean systemic arterial pressure after ADM inhalation. Interestingly, they reported that aerosolized ADM reduced endothelin (ET)-1 mRNA in lung tissue and ET-1 protein expression in pulmonary arteries. ET-1 is a potent pulmonary vasoconstrictor [88] and plays an important role in pulmonary hypertension [89-93]. Thus, reduction of ET-1 expression may contribute to the effect of aerosolized ADM on pulmonary hypertension. Recently, we have investigated the effects of ADM inhalation on pulmonary hemodynamics and exercise capacity in patients with IPAH [94]. Inhalation of aerosolized ADM (10 μg/kg) produced a 22% decrease in pulmonary vascular resistance. However, neither systemic arterial pressure nor heart rate was altered. These results suggest that inhaled ADM improves hemodynamics with pulmonary selectivity. In addition, inhalation of ADM improved exercise capacity, as indicated by increased peak oxygen consumption during exercise. Although further studies are necessary to maximize the efficiency and reproducibility of pulmonary ADM delivery, inhalation of ADM may be a promising approach to treat pulmonary hypertension without affecting the systemic circulation.

Recently, stem or progenitor cell transplantation has received much attention as a novel therapeutic option to regenerate a variety of tissues. In 1997, Asahara *et al.* isolated endothelial progenitor cells (EPC) from human peripheral blood [95]. EPC are mobilized from bone marrow into the peripheral blood in response to tissue ischemia or traumatic injury, migrate to sites of injured endothelium, and differentiate into mature endothelial cells *in situ* [96-98]. Transplantation of EPC has been shown to induce therapeutic angiogenesis in the ischemic heart or limb [99-101]. We have shown that intravenously administered EPC are incorporated into the pulmonary vasculature and attenuate pulmonary hypertension in the rat monocrotaline model of IPAH [102]. Thus, the regeneration of pulmonary vascular endothelium by cell transplantation may be a new therapeutic strategy for the treatment of pulmonary hypertension. A recent study has reported that human telomerase reverse transcriptase gene transfer enhances the angiogenic properties of EPC [103]. Considering the variety of protective effects of ADM on vascular endothelial cells, we hypothesized that ADM gene transfer into EPC may strengthen their therapeutic potential. Recently, Fukunaka *et al.* have developed a nonviral vector, gelatin hydrogel [104,105]. Positively charged gelatin can hold negatively charged plasmid DNA in its lattice structure. DNA-gelatin complexes can delay gene degradation, leading to efficient gene transfer [106,107]. Interestingly, EPC phagocytose ionically linked DNA-gelatin complexes in coculture, which allows nonviral gene transfer into EPC with high efficiency. ADM gene transfer into EPC inhibits cell apoptosis and induces proliferation and migration, suggesting that ADM gene transfer strengthens the therapeutic potential of EPC. Furthermore, genetically modified EPCs markedly secreted ADM peptide and ADM overproduction lasted for more than 16 days. Therefore, transplanted EPC may serve not only as a tissue-engineering tool to reconstruct the pulmonary vasculature but also as a vehicle for gene delivery to injured pulmonary endothelium. We have investigated whether cell (EPC)-based ADM gene transfer causes further improvement in monocrotaline-induced pulmonary hypertension in rats [102]. ADM gene-modified EPC were similarly incorporated into the pulmonary vasculature, and significantly decreased pulmonary vascular resistance compared with EPC alone. A single transplantation of ADM gene-modified EPC improved survival in monocrotaline rats compared with transplantation of EPC alone. Thus, a novel hybrid cell-gene therapy may be a new therapeutic strategy for the treatment of pulmonary hypertension including IPAH. However, the initial success of gelatin-mediated ADM gene therapy reported here should be confirmed by long-term experiments, and extensive toxicity studies in animals are needed before clinical trials.

SUMMARY

This article describes the physiological significance of ADM and its therapeutic potential for the treatment of pulmonary hypertension. ADM has been shown to possess a variety of actions, including vasodilatory actions, regulation of cell growth and survival, and modulation of hormone secretion. Plasma ADM levels are elevated in patients with pulmonary hypertension in proportion to the clinical severity, and circulating ADM is partially metabolized in the lungs. Nevertheless, exogenously administered ADM induces hemodynamic improvement. These findings suggest that ADM may be a promising therapeutic agent for the treatment of pulmonary hypertension including IPAH. Further studies are necessary to evaluate the long-term efficacy and safety of ADM in patients with pulmonary hypertension.

ABBREVIATIONS

ADM = Adrenomedullin

cAMP = Cyclic adenosine 3',5'-monophosphate

EPC = Endothelial progenitor cell

ET = Endothelin

IPAH = Idiopathic pulmonary arterial hypertension

NO = Nitric oxide

PI3K = Phosphatidylinositol 3-kinase

PKA = Protein kinase A

REFERENCES

[1] Rich, S., Dantzker, D.R., Ayres, S.M., Bergofsky, E.H., Brundage, B.H., Detre, K.M., Fishman, A.P., Goldring, R.M., Groves, B.M., Koerner, S.K. Primary pulmonary hypertension. A national prospective study. *Ann. Intern. Med.*, **1987**, *107*, 216-223.

[2] D'Alonzo, G.E., Barst, R.J., Ayres, S.M., Bergofsky, E.H., Brundage, B.H., Detre, K.M., Fishman, A.P., Goldring, R.M., Groves, B.M., Kernis, J.T. Survival in patients with primary pulmonary hypertension. Results from a national prospective registry. *Ann. Intern. Med.*, **1991**, *115*, 343-349.

[3] Archer, S., Rich, S. Primary pulmonary hypertension: a vascular biology and translational research "Work in progress". *Circulation*, **2000**, *102*, 2781-2791.

[4] Barst, R.J., Rubin, L.J., Long, W.A, McGoon, M.D., Rich, S., Badesch, D.B., Groves, B.M., Tapson, V.F., Bourge, R.C., Brundage, B.H. A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension. The Primary Pulmonary Hypertension Study Group. *N. Engl. J. Med.*, **1996**, *334*, 296-302.

[5] McLaughlin, V.V., Genthner, D.E., Panella, M.M., Rich, S. Reduction in pulmonary vascular resistance with long-term epoprostenol (prostacyclin) therapy in primary pulmonary hypertension. *N. Engl. J. Med.*, **1998**, *338*, 273-277.

[6] Rubin, L.J., Mendoza, J., Hood, M., McGoon, M., Barst, R., Williams, W.B., Diehl, J.H., Crow, J., Long, W. Treatment of primary pulmonary hypertension with continuous intravenous prostacyclin (epoprostenol). Results of a randomized trial. *Ann. Intern. Med.*, **1990**, *112*, 485-491.

[7] Reitz, B.A., Wallwork, J.L., Hunt, S.A., Pennock, J.L., Billingham, M.E., Oyer, P.E., Stinson, E.B., Shumway, N.E. Heart-lung transplantation: successful therapy for patients with pulmonary vascular disease. *N. Engl. J. Med.*, **1982**, *306*, 57-64.

[8] Pasque, M.K., Trulock, E.P., Kaiser, L.R, Cooper, J.D. Single-lung transplantation for pulmonary hypertension. Three-month hemodynamic follow-up. *Circulation*, **1991**, *84*, 2275-2279.

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

[10] McLatchie, L.M., Fraser, N.J., Main, M.J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M.G., Foord, S.M. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature*, **1998**, *393*, 333-339.

[11] Ichiki, Y., Kitamura, K., Kangawa, K., Kawamoto, M., Matsuo, H., Eto, T. Distribution and characterization of immunoreactive adrenomedullin in human tissue and plasma. *FEBS Lett.*, **1994**, *338*, 6-10.

[12] Sakata, J., Shimokubo, T., Kitamura, K., Nishizono, M., Ichiki, Y., Kangawa, K., Matsuo, H., Eto, T. Distribution and characterization of immunoreactive rat adrenomedullin in tissue and plasma. *FEBS Lett.*, **1994**, *352*, 105-108.

[13] Shimekake, Y., Nagata, K., Ohta, S., Kambayashi, Y., Teraoka, H., Kitamura, K., Eto, T., Kangawa, K., Matsuo, H. Adrenomedullin stimulates two signal transduction pathways, cAMP accumulation and Ca^{2+} mobilization, in bovine aortic endothelial cells. *J. Biol. Chem.*, **1995**, *270*, 4412-4417.

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

[15] Sugo, S., Minamino, N., Shoji, H., Kangawa, K., Kitamura, K., Eto, T., Matsuo, H. Production and secretion of adrenomedullin from vascular smooth muscle cells: augmented production by tumor necrosis factor-alpha. *Biochem. Biophys. Res. Commun.*, **1994**, *203*, 719-726.

[16] Majid, D.S., Kadowitz, P.J., Coy, D.H., Navar, L.G. Renal responses to intra-arterial administration of adrenomedullin in dogs. *Am .J .Physiol.*, **1996**, *270*, F200-F205.

[17] Parkes, D.G., May, C.N. Direct cardiac and vascular actions of adrenomedullin in conscious sheep. *Br. J. Pharmacol.*, **1997**, *120*, 1179-1185.

[18] Szokodi, I., Kinnunen, P., Tavi, P., Weckstrom, M., Toth, M., Ruskoaho, H. Evidence for cAMP-independent mechanisms mediating the effects of adrenomedullin, a new inotropic peptide. *Circulation*, **1998**, *97*, 1062-70.

[19] Nagaya, N., Goto, Y., Satoh, T., Sumida, H., Kojima, S., Miyatake, K., Kangawa, K. Intravenous adrenomedullin in myocardial function and energy metabolism in patients after myocardial infarction. *J. Cardiovasc. Pharmacol.*, **2002**, *39*, 754-760.

[20] Kato, H., Shichiri, M., Marumo, F., Hirata, Y. Adrenomedullin as an autocrine/paracrine apoptosis survival factor for rat endothelial cells. *Endocrinology*, **1997**, *138*, 2615-2620.

[21] Nagaya, N., Mori, H., Murakami, S., Kangawa, K., Kitamura, S. Adrenomedullin: angiogenesis and gene therapy. *Am. J. Physiol. Regul. Integr. Comp .Physiol.*, (in press).

[22] Hanabusa, K., Nagaya, N., Iwase, T., Itoh, T., Murakami, S., Shimizu, Y., Taki, W., Miyatake, K., Kangawa, K. Adrenomedullin enhances therapeutic potency of mesenchymal stem cells after experimental stroke in rats. *Stroke*. **2005**, *36*, 853-858.

[23] Iwase, T., Nagaya, N., Fujii, T., Itoh, T., Ishibashi-Ueda, H., Yamagishi, M., Miyatake, K., Matsumoto, T., Kitamura, S., Kangawa, K. Adrenomedullin enhances angiogenic potency of bone marrow transplantation in a rat model of hindlimb ischemia. *Circulation*, **2005**, *111*, 356-362.

[24] Fujii, T., Nagaya, N., Iwase, T., Murakami, S., Miyahara, Y., Nishigami, K., Ishibashi-Ueda H., Shirai, M., Itoh, T., Ishino, K., Sano, S., Kangawa, K., Mori, H. Adrenomedullin enhances therapeutic potency of bone marrow transplantation for myocardial infarction in rats. *Am. J. Physiol. Heart. Circ. Physiol.*, **2005**, *288*, H1444-H1450.

[25] Okumura, H., Nagaya, N., Itoh, T., Okano, I., Hino, J., Mori, K., Tsukamoto, Y., Ishibashi-Ueda, H., Miwa, S., Tambara, K., Toyokuni, S., Yutani, C., Kangawa, K. Adrenomedullin infusion attenuates myocardial ischemia/reperfusion injury through the phosphatidylinositol 3-kinase/Akt-dependent pathway. *Circulation*, **2004**, *109*, 242-248.

[26] Kohno, M., Hanehira, T., Kano, H., Horio, T., Yokokawa, K., Ikeda, M., Minami, M., Yasunari, K., Yoshikawa, J. Plasma adrenomedullin concentrations in essential hypertension. *Hypertension*, **1996**, *27*, 102-107.

[27] Nishikimi, T., Yoshihara, F., Kanazawa, A., Okano, I., Horio, T., Nagaya, N., Yutani, C., Matsuo, H., Matsuoka, H., Kangawa, K. Role of increased circulating and renal adrenomedullin in rats with malignant hypertension. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*; **2001**, *281*, R2079-R2087.

[28] Nishikimi, T., Horio, T., Kohmoto, Y., Yoshihara, F., Nagaya, N., Inenaga, T., Saito, M., Teranishi, M., Nakamura, M., Ohrui, M., Kawano, Y., Matsuo, H., Ishimitsu, T., Takishita, S., Matsuoka, H., Kangawa, K. Molecular forms of plasma and urinary adrenomedullin in normal, essential hypertension and chronic renal failure. *J. Hypertens.*, **2001**, *19*, 765-773.

[29] Kobayashi, K., Kitamura, K., Hirayama, N., Date, H., Kashiwagi, T., Ikushima, I., Hanada, Y., Nagatomo, Y., Takenaga, M., Ishikawa, T., Imamura, T., Koiwaya, Y., Eto, T. Increased plasma adrenomedullin in acute myocardial infarction. *Am .Heart J.*, **1996**, *131*, 676-680.

[30] Miyao, Y., Nishikimi, T., Goto, Y., Miyazaki, S., Daikoku, S., Morii, I., Matsumoto, T., Takishita, S., Miyata, A., Matsuo, H., Kangawa, K., Nonogi, H. Increased plasma adrenomedullin levels in patients with acute myocardial infarction in proportion to the clinical severity. *Heart*, **1998**, *79*, 39-44.

[31] Nagaya, N., Nishikimi, T., Yoshihara, F., Horio, T., Morimoto, A., Kangawa, K. Cardiac adrenomedullin gene expression and peptide accumulation after acute myocardial infarction in rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **2000**, *278*, R1019-R1026.

[32] Nagaya, N., Nishikimi, T., Uematsu, M., Yoshitomi, Y., Miyao, Y., Miyazaki, S., Goto, Y., Kojima, S., Kuramochi, M., Matsuo, H., Kangawa, K., Nonogi, H. Plasma adrenomedullin as an indicator of prognosis after acute myocardial infarction. *Heart*, 1999, *81*, 483-487.

[33] Jougasaki, M., Wei, C.M., McKinley, L.J., Burnett, J.C.Jr., Elevation of circulating and ventricular adrenomedullin in human congestive heart failure. *Circulation*, **1995**, *92*, 286-289.

[34] Nishikimi, T., Saito, Y., Kitamura, K., Ishimitsu, T., Eto, T., Kangawa, K., Matsuo, H., Omae, T., Matsuoka, H. Increased plasma levels of adrenomedullin in patients with heart failure. *J. Am. Coll .Cardiol.*, **1995**, *26*, 1424-1431.

[35] Watanabe, K., Nishikimi, T., Takamuro, M., Yasuda, K., Ishikawa, Y., Tanabe, S., Yamada, O., Nagaya, N., Matsuoka, H., Kangawa, K., Echigo, S. Two molecular forms of adrenomedullin in congenital heart disease. *Pediatr. Cardiol.*, **2003**, *24*, 559-565.

[36] Tambara, K., Fujita, M., Nagaya, N., Miyamoto, S., Iwakura, A., Doi, K., Sakaguchi, G., Nishimura, K., Kangawa, K., Komeda, M. Increased pericardial fluid concentrations of the mature form of adrenomedullin in patients with cardiac remodelling. *Heart,* **2002**, *87*, 242-246.

[37] Yoshihara, F., Nishikimi, T., Horio, T., Yutani, C., Nagaya ,N., Matsuo, H., Ohe, T., Kangawa, K. Ventricular adrenomedullin concentration is a sensitive biochemical marker for volume and pressure overload in rats. *Am. J. Physiol. Heart. Circ. Physiol.*, **2000**, *278*, H633-H642.

[38] Morimoto, A., Nishikimi, T., Yoshihara, F., Horio, T., Nagaya, N., Matsuo, H., Dohi, K., Kangawa, K. Ventricular adrenomedullin levels correlate with the extent of cardiac hypertrophy in rats. *Hypertension,* **1999**, *33*, 1146-1152.

[39] Nagaya, N., Nishikimi, T., Horio, T., Yoshihara, F., Kanazawa, A., Matsuo, H., Kangawa, K. Cardiovascular and renal effects of adrenomedullin in rats with heart failure. *Am. J. Physiol.*, **1999**, *276*, R213-R218.

[40] Ishimitsu, T., Nishikimi, T., Saito, Y., Kitamura, K., Eto, T., Kangawa, K., Matsuo, H., Omae, T., Matsuoka, H. Plasma levels of adrenomedullin, a newly identified hypotensive peptide, in patients with hypertension and renal failure. *J. Clin. Invest.*, **1994**, *94*, 2158-2161.

[41] Kakishita, M., Nishikimi, T., Okano, Y., Satoh, T., Kyotani, S., Nagaya, N., Fukushima, K., Nakanishi, N., Takishita, S., Miyata, A., Kangawa, K., Matsuo, H., Kunieda, T. Increased plasma levels of adrenomedullin in patients with pulmonary hypertension. *Clin. Sci., (Lond)* **1999**, *96*, 33-39.

[42] Vijay, P., Szekely, L., Sharp, T.G., Miller, A., Bando, K., Brown, J.W. Adrenomedullin in patients at high risk for pulmonary hypertension. *Ann. Thorac. Surg.*, **1998**, *66*, 500-505.

[43] Nishikimi, T., Nagata, S., Sasaki, T., Yoshihara, F., Nagaya, N., Horio, T., Matsuo, H., Matsuoka, H., Kangawa, K. The active molecular form of plasma adrenomedullin is extracted in the pulmonary circulation in patients with mitral stenosis: possible role of adrenomedullin in pulmonary hypertension. *Clin. Sci (Lond).*, **2001**, *100*, 61-66.

[44] Shimosawa, T., Shibagaki, Y., Ishibashi, K., Kitamura, K., Kangawa, K., Kato, S., Ando, K., Fujita, T. Adrenomedullin, an endogenous peptide, counteracts cardiovascular damage. *Circulation,* **2002**, *105*, 106-111.

[45] Niu, P., Shindo, T., Iwata, H., Iimuro, S., Takeda, N., Zhang, Y., Ebihara, A., Suematsu, Y., Kangawa, K., Hirata, Y., Nagai, R. Protective effects of endogenous adrenomedullin on cardiac hypertrophy, fibrosis, and renal damage. *Circulation,* **2004**, *109*, 1789-1794.

[46] Yoshibayashi, M., Kamiya, T., Kitamura, K., Saito, Y., Kangawa, K., Nishikimi, T., Matsuoka, H., Eto, T., Matsuo, H. Plasma levels of adrenomedullin in primary and secondary pulmonary hypertension in patients <20 years of age. *Am. J. Cardiol.*, **1997**, *79*, 1556-1558.

[47] Shimokubo, T., Sakata, J., Kitamura, K., Kangawa, K., Matsuo, H., Eto, T. Augmented adrenomedullin concentrations in right ventricle and plasma of experimental pulmonary hypertension. *Life Sci.*, **1995**, *57*, 1771-1779.

[48] Kitamura, K., Sakata, J., Kangawa, K., Kojima, M., Matsuo, H., Eto, T. Cloning and characterization of cDNA encoding a precursor for human adrenomedullin. *Biochem. Biophys. Res. Commun.*, **1993**, *194*, 720-725.

[49] Nishikimi, T., Kitamura, K., Saito, Y., Shimada, K., Ishimitsu, T., Takamiya, M., Kangawa, K., Matsuo, H., Eto, T., Omae, T. Clinical studies on the sites of production and clearance of circulating adrenomedullin in human subjects. *Hypertension,* **1994**, *24*, 600-604.

[50] Nakayama, M., Takahashi, K., Murakami, O., Shirato, K., Shibahara, S. Induction of adrenomedullin by hypoxia and cobalt chloride in human colorectal carcinoma cells. *Biochem. Biophys. Res. Commun.*, **1998**, *243*, 514-517.

[51] Sugo, S., Minamino, N., Shoji, H., Kangawa, K., Kitamura, K., Eto, T., Matsuo, H. Interleukin-1, tumor necrosis factor and lipopolysaccharide additively stimulate production of adrenomedullin in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **1995**, *207*, 25-32.

[52] Chun, T.H., Itoh, H., Ogawa, Y., Tamura, N., Takaya, K., Igaki, T., Yamashita, J., Doi, K., Inoue, M., Masatsugu, K., Korenaga, R., Ando, J., Nakao, K. Shear stress augments expression of C-type natriuretic peptide and adrenomedullin. *Hypert.,* **1997**, *29*, 1296-1302.

[53] Garayoa, M., Martinez, A., Lee, S., Pio, R., An, W.G., Neckers, L., Trepel, J., Montuenga, L.M., Ryan, H., Johnson, R., Gassmann, M., Cuttitta, F. Hypoxia-inducible factor-1 (HIF-1) up-regulates adrenomedullin expression in human tumor cell lines during oxygen deprivation: a possible promotion mechanism of carcinogenesis. *Mol. Endocrinol.*, **2000**, *14*, 848-862.

[54] Champion, H.C., Akers, D.L., Santiago, J.A., Lambert. D.G., McNamara, D.B., Kadowitz PJ. Analysis of responses to human synthetic adrenomedullin and calcitonin gene-related peptides in the hindlimb vascular bed of the cat. *Mol. Cell Biochem.*, **1997**, *176*, 5-11.

[55] Shirai, M., Shimouchi, A., Ikeda, S., Ninomiya, I., Sunagawa, K., Kangawa, K., Matsuo, H. Vasodilator effects of adrenomedullin on small pulmonary arteries and veins in anaesthetized cats. *Br. J. Pharmacol.*, **1997**, *121*, 679-686.

[56] Eguchi, S., Hirata, Y., Kano, H., Sato, K., Watanabe, Y., Watanabe, T.X., Nakajima, K., Sakakibara, S., Marumo, F. Specific receptors for adrenomedullin in cultured rat vascular smooth muscle cells. *FEBS Lett.*, **1994**, *340*, 226-230.

[57] Ishizaka, Y., Ishizaka, Y., Tanaka, M., Kitamura, K., Kangawa, K., Minamino, N., Matsuo, H., Eto, T. Adrenomedullin stimulates cyclic AMP formation in rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **1994**, *200*, 642-646.

[58] Feng, C.J., Kang, B., Kaye, A.D., Kadowitz, P.J, Nossaman, B.D. L-NAME modulates responses to adrenomedullin in the hindquarters vascular bed of the rat. *Life. Sci.*,**1994**, *55*, L433-L438.

[59] Venema, R.C., Sayegh, H.S., Kent, J.D., Harrison, D.G.. Identification, characterization, and comparison of the calmodulin-binding domains of the endothelial and inducible nitric oxide synthases. *J. Biol. Chem.*, **1996**, *271*, 6435-6440.

[60] Nishimatsu, H., Suzuki, E., Nagata, D., Moriyama, N., Satonaka, H., Walsh, K., Sata, M., Kangawa, K., Matsuo, H., Goto, A., Kitamura, T., Hirata, Y. Adrenomedullin induces endothelium-dependent vasorelaxation *via* the phosphatidylinositol 3-kinase/Akt-dependent pathway in rat aorta. *Circ. Res.*, **2001**, *89*, 63-70.

[61] Owji, A.A., Smith, D.M., Coppock, H.A., Morgan, D.G., Bhogal, R., Ghatei, M.A., Bloom, S.R. An abundant and specific binding site for the novel vasodilator adrenomedullin in the rat. *Endocrinology,* **1995**, *136*, 2127-2134.

[62] Lippton, H., Chang, J.K., Hao, Q., Summer, W., Hyman, A.L. Adrenomedullin dilates the pulmonary vascular bed *in vivo*. *J. Appl. Physiol.*, **1994**, *76*, 2154-2156.

[63] Christman, B.W., McPherson, C.D., Newman, J.H., King, G.A., Bernard, G.R., Groves, B.M., Loyd, J.E. An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *N. Engl. J. Med.*, **1992**, *327*, 70-75.

[64] Tuder, R.M., Cool, C.D., Geraci, M.W., Wang, J., Abman ,S.H., Wright, L., Badesch, D., Voelkel, N.F. Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. *Am. J. Respir. Crit. Care. Med.*, **1999**, *159*, 1925-1932.

[65] Giaid, A., Saleh, D. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med.*, **1995**, *333*, 214-221.

[66] Sata, M., Kakoki, M., Nagata, D., Nishimatsu, H., Suzuki, E., Aoyagi, T., Sugiura, S., Kojima, H., Nagano, T., Kangawa, K., Matsuo, H., Omata, M., Nagai, R., Hirata, Y. Adrenomedullin and nitric oxide inhibit human endothelial cell apoptosis *via* a cyclic GMP-independent mechanism. *Hypertension,* **2000**, *36*, 83-88.

[67] Kim, W., Moon, S.O., Sung, M.J., Kim, S.H., Lee, S., So, J.N., Park, S.K. Angiogenic role of adrenomedullin through activation of Akt, mitogen-activated protein kinase, and focal adhesion kinase in endothelial cells. *FASEB J.*, **2003**, *17*, 1937-1939.

[68] Pietra, G.G., Edwards, W.D., Kay, J.M., Rich, S., Kernis, J., Schloo, B., Ayres, S.M., Bergofsky, E.H., Brundage, B.H., Detre, K.M. Histopathology of primary pulmonary hypertension. A

qualitative and quantitative study of pulmonary blood vessels from 58 patients in the National Heart, Lung, and Blood Institute, Primary Pulmonary Hypertension Registry. *Circulation,* **1989**, *80*, 1198-1206.

[69] Tuder, R.M., Groves, B., Badesch, D.B., Voelkel NF. Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *Am.J. Pathol.*, **1994**, *144*, 275-285.

[70] Kano, H., Kohno, M., Yasunari, K., Yokokawa, K., Horio, T., Ikeda, M., Minami, M., Hanehira, T., Takeda, T., Yoshikawa, J. Adrenomedullin as a novel antiproliferative factor of vascular smooth muscle cells. *J. Hypertens.*, **1996**, *14*, 209-213.

[71] Upton, P.D., Wharton, J., Coppock, H., Davie, N., Yang, X., Yacoub, M.H., Ghatei, M.A., Polak, J.M., Bloom, S.R., Smith, D.M., Morrell, N.W. Adrenomedullin expression and growth inhibitory effects in distinct pulmonary artery smooth muscle cell subpopulations. *Am. J. Respir .Cell. Mol. Biol.*, **2001**, *24*, 170-178.

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

[73] Yoshihara, F., Nishikimi, T., Horio, T., Yutani, C., Takishita, S., Matsuo, H., Ohe, T., Kangawa, K. Chronic infusion of adrenomedullin reduces pulmonary hypertension and lessens right ventricular hypertrophy in rats administered monocrotaline. *Eur. J. Pharmacol.*, **1998**, *355*, 33-39.

[74] Chini, E.N., Chini, C.C., Bolliger, C., Jougasaki, M., Grande, J.P., Burnett, J.C., Jr, Dousa ,T.P. Cytoprotective effects of adrenomedullin in glomerular cell injury: central role of cAMP signaling pathway. *Kidney. Int.*, **1997**, *52*, 917-25.

[75] Matsui, H., Shimosawa, T., Itakura, K., Guanqun, X., Ando, K., Fujita, T. Adrenomedullin can protect against pulmonary vascular remodeling induced by hypoxia. *Circulation,* **2004**, *109*, 2246-2251.

[76] Heaton, J., Lin, B., Chang, J.K., Steinberg, S., Hyman, A., Lippton, H. Pulmonary vasodilation to adrenomedullin: a novel peptide in humans. *Am .J. Physiol.*, **1995**, *268*, H2211-H2215.

[77] Yang, B.C., Lippton, H., Gumusel, B., Hyman, A., Mehta, J.L. Adrenomedullin dilates rat pulmonary artery rings during hypoxia: role of nitric oxide and vasodilator prostaglandins. *J .Cardiovasc. Pharmacol.*, **1996**, *28*, 458-462.

[78] Rademaker, M.T., Charles, C.J., Lewis, L.K., Yandle, T.G., Cooper, G.J., Coy, D.H., Richards, A.M., Nicholls, M.G. Beneficial hemodynamic and renal effects of adrenomedullin in an ovine model of heart failure. *Circulation,* **1997**, *96*, 1983-1990.

[79] Nagaya, N., Satoh, T., Nishikimi, T., Uematsu, M., Furuichi, S., Sakamaki, F., Oya, H., Kyotani, S., Nakanishi, N., Goto, Y., Masuda, Y., Miyatake, K., Kangawa, K. Hemodynamic, renal, and hormonal effects of adrenomedullin infusion in patients with congestive heart failure. *Circulation,* **2000**, *101*, 498-503.

[80] Nagaya, N., Nishikimi, T., Uematsu, M., Satoh, T., Oya, H., Kyotani, S., Sakamaki, F., Ueno, K., Nakanishi, N., Miyatake, K., Kangawa, K. Haemodynamic and hormonal effects of adrenomedullin in patients with pulmonary hypertension. *Heart,* **2000**, *84*, 653-658.

[81] Nishikimi, T., Horio, T., Yoshihara, F., Nagaya, N., Matsuo, H., Kangawa, K. Effect of adrenomedullin on cAMP and cGMP levels in rat cardiac myocytes and nonmyocytes. *Eur. J. Pharmacol.*, **1998**, *353*, 337-344.

[82] Ihara, T., Ikeda, U., Tate, Y., Ishibashi, S., Shimada, K. Positive inotropic effects of adrenomedullin on rat papillary muscle. *Eur. J. Pharmacol.*, **2000**, *390*, 167-172.

[83] Szokodi, I., Kinnunen, P., Tavi, P., Weckstrom, M., Toth, M., Ruskoaho, H. Evidence for cAMP-independent mechanisms mediating the effects of adrenomedullin, a new inotropic peptide. *Circulation,* **1998**, *97*, 1062-1070.

[84] Hoeper, M.M., Schwarze, M., Ehlerding, S., Adler-Schuermeyer, A., Spiekerkoetter, E., Niedermeyer, J., Hamm, M., Fabel, H. Long-term treatment of primary pulmonary hypertension with aerosolized iloprost, a prostacyclin analogue. *N. Engl. J. Med.*, **2000**, *342*, 1866-1870.

[85] Walmrath, D., Schneider, T., Pilch, J., Grimminger, F., Seeger, W. Aerosolised prostacyclin in adult respiratory distress syndrome. *Lancet,* **1993**, *342*, 961-962.

[86] Nagaya, N., Okumura, H., Uematsu, M., Shimizu, W., Ono, F., Shirai, M., Mori, H., Miyatake, K., Kangawa, K. Repeated inhalation of adrenomedullin ameliorates pulmonary hypertension and survival in monocrotaline rats. *Am. J. Physiol. Heart. Circ. Physiol.*, **2003**, *285*, H2125-H2131.

[87] von der Hardt, K., Kandler, M.A., Chada, M., Cubra, A., Schoof, E., Amann, K., Rascher, W., Dotsch J. Brief adrenomedullin inhalation leads to sustained reduction of pulmonary artery pressure. *Eur. Respir. J.*, **2004**, *24*, 615-623.

[88] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., Masaki, T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature,* **1988**, *332*, 411-415.

[89] Stewart, D.J., Levy, R.D., Cernacek, P., Langleben D. Increased plasma endothelin-1 in pulmonary hypertension: marker or mediator of disease? *Ann. Intern. Med.*, **1991**, *114*, 464-469.

[90] Nootens, M., Kaufmann, E., Rector, T., Toher, C., Judd, D., Francis, G.S., Rich, S. Neurohormonal activation in patients with right ventricular failure from pulmonary hypertension: relation to hemodynamic variables and endothelin levels. *J. Am. Coll. Cardiol.*, **1995**, *26*, 1581-1585.

[91] Rubens, C., Ewert, R., Halank, M., Wensel, R., Orzechowski, H.D., Schultheiss, H.P., Hoeffken G. Big endothelin-1 and endothelin-1 plasma levels are correlated with the severity of primary pulmonary hypertension. *Chest,* **2001**, *120*, 1562-1569.

[92] Collados, M.T., Velazquez, B., Borbolla, J.R., Sandoval, J., Masso, F., Montano, L.F., Guarner V. Endothelin-1 and functional tissue factor: a possible relationship with severity in primary pulmonary hypertension. *Heart Vessels,* **2003**, *18*, 12-17.

[93] Giaid, A., Yanagisawa, M., Langleben, D., Michel, R.P., Levy, R., Shennib, H., Kimura, S., Masaki, T., Duguid, W.P., Stewart, D.J. Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med.*, **1993**, *328*, 1732-1739.

[94] Nagaya, N., Kyotani, S., Uematsu, M., Ueno, K., Oya, H., Nakanishi, N., Shirai, M., Mori, H., Miyatake, K., Kangawa, K. Effects of adrenomedullin inhalation on hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension. *Circulation,* **2004**, *109*, 351-356.

[95] Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science,* **1997**, *275*, 964-967.

[96] Takahashi, T., Kalka, C., Masuda, H., Chen, D., Silver, M., Kearney, M., Magner, M., Isner, J.M., Asahara, T. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat. Med.*, **1999**, *5*, 434-438.

[97] Gill, M., Dias, S., Hattori, K., Rivera, M.L., Hicklin, D., Witte, L., Girardi, L., Yurt, R., Himel, H., Rafii, S. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. *Circ. Res.*, **2001**, *88*, 167-174.

[98] Kawamoto, A., Gwon, H.C., Iwaguro, H., Yamaguchi, J.I., Uchida, S., Masuda, H., Silver, M., Ma, H., Kearney, M., Isner, J.M., Asahara, T. Therapeutic potential of *ex vivo* expanded endothelial progenitor cells for myocardial ischemia. *Circulation,* **2001**, *103*, 634-637.

[99] Murohara, T., Ikeda, H., Duan, J., Shintani, S., Sasaki, K., Eguchi, H., Onitsuka, I., Matsui, K., Imaizumi, T. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. *J .Clin. Invest.*, **2000**, *105*, 1527-1536.

[100] Shintani, S., Murohara, T., Ikeda, H., Ueno, T., Sasaki, K., Duan, J., Imaizumi, T.,Augmentation of postnatal neovascularization with autologous bone marrow transplantation. *Circulation,* **2001**, *103*, 897-903.

[101] Tateishi-Yuyama. E., Matsubara, H., Murohara. T., Ikeda, U., Shintani, S., Masaki, H., Amano, K., Kishimoto, Y., Yoshimoto, K., Akashi, H., Shimada, K., Iwasaka, T., Imaizumi, T. Therapeutic Angiogenesis using Cell Transplantation (TACT) Study Investigators. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet,* **2002**, *360*, 427-435.

[102] Nagaya, N., Kangawa, K., Kanda, M., Uematsu, M., Horio, T., Fukuyama, N., Hino. J., Harada-Shiba, M., Okumura, H., Tabata, Y., Mochizuki, N., Chiba, Y., Nishioka, K., Miyatake., K., Asahara, T., Hara, H., Mori, H. Hybrid cell-gene therapy for

pulmonary hypertension based on phagocytosing action of endothelial progenitor cells. *Circulation,* **2003,** *108*, 889-895.

[103] Murasawa, S., Llevadot, J., Silver, M., Isner, J.M., Losordo, D.W., Asahara, T. Constitutive human telomerase reverse transcriptase expression enhances regenerative properties of endothelial progenitor cells. *Circulation,* **2002,** *106*, 1133-1139.

[104] Fukunaka, Y., Iwanaga, K., Morimoto, K., Kakemi, M., Tabata, Y. Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. *J. Control. Release,* **2002,** *80*, 333-343.

[105] Kushibiki, T., Tomoshige, R., Fukunaka, Y., Kakemi, M., Tabata, Y. *In vivo* release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents. *J. Cont. Release,* **2003,** *90*, 207-216.

[106] Kasahara, H., Tanaka, E., Fukuyama, N., Sato, E., Sakamoto, H., Tabata, Y., Ando, K., Iseki, H., Shinozaki, Y., Kimura, K., Kuwabara, E., Koide, S., Nakazawa, H., Mori, H. Biodegradable gelatin hydrogel potentiates the angiogenic effect of fibroblast growth factor 4 plasmid in rabbit hindlimb ischemia. *J. Am. Coll. Cardiol.*, **2003,** *41*, 1056-1062.

[107] Tokunaga, N., Nagaya, N., Shirai, M., Tanaka, E., Ishibashi-Ueda, H., Harada-Shiba, M., Kanda, M., Ito, T., Shimizu, W., Tabata, Y., Uematsu, M., Nishigami, K., Sano, S., Kangawa, K., Mori ,H. Adrenomedullin gene transfer induces therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia: benefits of a novel nonviral vector, gelatin. *Circulation,* **2004,** *109*, 526-531.

Received: 18 April, 2005 Revised: 17 January, 2006 Accepted: 16 February, 2006

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart Association®

Learn and Live℠

Inflammatory Response to Acute Myocardial Infarction Augments Neointimal Hyperplasia After Vascular Injury in a Remote Artery

Minoru Takaoka, Shiro Uemura, Hiroyuki Kawata, Kei-ichi Imagawa, Yukiji Takeda, Kimihiko Nakatani, Noriyuki Naya, Manabu Horii, Shigeru Yamano, Yoshihiro Miyamoto, Yasunao Yoshimasa and Yoshihiko Saito

Arterioscler. Thromb. Vasc. Biol. 2006;26;2083-2089; originally published online Jun 15, 2006;
DOI: 10.1161/01.ATV.0000232528.93786.0a
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 1079-5642. Online ISSN: 1524-4636

Inflammatory Response to Acute Myocardial Infarction Augments Neointimal Hyperplasia After Vascular Injury in a Remote Artery

Minoru Takaoka, Shiro Uemura, Hiroyuki Kawata, Kei-ichi Imagawa, Yukiji Takeda, Kimihiko Nakatani, Noriyuki Naya, Manabu Horii, Shigeru Yamano, Yoshihiro Miyamoto, Yasunao Yoshimasa, Yoshihiko Saito

Objective—Percutaneous coronary intervention (PCI) is currently the most widely accepted treatment for acute myocardial infarction (AMI). It remains unclear, however, whether post-AMI conditions might exacerbate neointimal hyperplasia and restenosis following PCI. Given that both a medial smooth muscle cell lineage and a bone marrow (BM)-derived hematopoietic stem cell lineage are now thought to contribute to neointima formation, the primary aims of the present study were to determine whether AMI augments neointimal hyperplasia at sites of arterial injury, and whether BM-derived cells contribute to that process.

Methods and Results—We simultaneously generated models of AMI and arterial injury in the same mice, some of which had received BM transplantation. We found that AMI augments neointimal hyperplasia at sites of femoral artery injury by ≈35% ($P<0.05$), but that while BM-derived cells contributed to neointimal hyperplasia, they did not contribute to the AMI-related augmentation. Expression of interleukin (IL)-6 mRNA was ≈7-fold higher in the neointimas of mice subjected to both AMI and arterial injury than in those of mice subjected to arterial injury alone. In addition, we observed increased synthesis of tumor necrosis factor (TNF)-α within infarcted hearts and TNF-α receptor type 1 (TNFR1) within injured arteries. Chronic treatment with pentoxifylline, which mainly inhibits TNF-α synthesis, reduced levels of circulating TNF-α and attenuated neointimal hyperplasia after AMI.

Conclusions—Conditions after AMI could exacerbate postangioplasty restenosis, not by increasing mobilization of BM-derived cells, but by stimulating signaling via TNF-α, TNFR1 and IL-6. (***Arterioscler Thromb Vasc Biol.* 2006; 26:2083-2089.**)

Key Words: bone marrow ■ inflammation ■ myocardial infarction ■ restenosis ■ smooth muscle cell

Both the occurrence and eventual healing of acute myocardial infarction (AMI) evoke inflammatory processes that lead to clinical components of instability, as evidenced by the high rate of subsequent coronary artery events, including recurrent MI and in-stent restenosis after percutaneous coronary intervention (PCI).[1–3] It is well known from both experimental and clinical observations that local upregulation of the expression of proinflammatory cytokines in activated smooth muscle cells (SMCs) contributes significantly to restenosis after balloon angioplasty and stent implantation.[4,5] In the setting of AMI, moreover, various proinflammatory cytokines and growth factors, including TNF-α, IL-1β, IL-6 and vascular endothelial growth factor (VEGF), are expressed in both infarcted and noninfarcted regions of the heart, and their plasma levels are elevated for ≈2 weeks after AMI,[6–10] raising the possibility that they, too, contribute to neointimal hyperplasia after PCI.

Recent findings suggest that 2 lineages of neointimal SMCs are involved in vascular remodeling after injury: a medial SMC lineage whose activation is triggered by various proinflammatory cytokines (the classical scenario), and a newly identified bone marrow (BM)-derived hematopoietic stem cell lineage.[11,12] It now appears that hematopoietic stem cells and endothelial progenitor cells are released from BM into the peripheral circulation during the early phase of AMI.[13,14] Thus, the mechanism for AMI-related vascular remodeling is apparently more complex than was recognized before the emergence of these new findings.

Within that context, the first aim of the present study was to determine whether AMI is, itself, capable of promoting neointimal hyperplasia at distant sites of arterial injury, such as would be caused by PCI. If so, the second aim of this study was to determine whether BM-derived cells contribute to that process. To accomplish these aims, we simultaneously gen-

Original received May 30, 2005; final version accepted April 23, 2006.

From the First Department of Internal Medicine (M.T., S.U., H.K., K.I., Y.T., K.N., N.N., M.H., S.Y.,Y.S.), Nara Medical University, Nara, Japan; National Cardiovascular Center (Y.M., Y.Y.), Suita, Osaka, Japan.

Correspondence to Yoshihiko Saito, First Department of Internal Medicine, Nara Medical University, 84 Shijo-cho, Kashihara, Nara 634-8522, Japan. E-mail yssaito@naramed-u.ac.jp

Arterioscler Thromb Vasc Biol. **is available at http://www.atvbaha.org** **DOI: 10.1161/01.ATV.0000232528.93786.0a**

erated experimental models of AMI and femoral arterial injury in the same mice, some of which had previously received BM transplantation (BMT) from green fluorescence protein (GFP) mice. Here we show that the inflammatory response to AMI augments neointimal hyperplasia in the injured femoral artery, but whereas BM-derived cells contribute to that neointima formation, they do not significantly contribute to the AMI-related augmentation of the response. Moreover, we demonstrate that the TNF-α synthesis inhibitor pentoxifylline (PTX)[15–17] reduces levels of circulating TNF-α and attenuates neointimal hyperplasia after AMI. Apparently, cross-talk between the heart and injured artery via signaling pathways mediated by inflammatory cytokines, especially TNF-α, TNF receptor type 1 (TNFR1) and IL-6, are involved in this process.

Materials and Methods

Animals

C57BL/6 mice were purchased from SLC (Shizuoka, Japan). Transgenic mice (C57BL/6 background) that ubiquitously express enhanced GFP (GFP mice) were a generous gift from Dr Masaru Okabe (Osaka University, Osaka, Japan).[18] All experimental procedures were performed in accordance with protocols approved by the Ethics Review Committee for Animal Experimentation of Nara Medical University and National Cardiovascular Center.

Bone Marrow Reconstitution

Bone marrow reconstitution (BMT) was performed as described previously.[11] One day after exposing 8-week-old male wild-type mice to a lethal dose (9.0 Gy) of X-irradiation, they received a tail vein injection of unfractionated BM cells (1×10^6) that had been harvested from the femora and tibias of GFP mice and suspended in 0.2 mL of phosphate-buffered saline. Eight weeks after BMT, peripheral leukocytes had been reconstituted to >90% of control, as determined by flow cytometry.

AMI

AMI was induced in mice as described previously.[19,20] The precise methods are described online only. Please see http://atvb.ahajournals.org.

Vascular Injury

Vascular injury (VI) was induced as described previously.[21] Mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), and the femoral artery was exposed. A straight spring wire (0.38 mm in diameter, No. C-SF-15 to 15; COOK, Bloomington, Ind) was then inserted into the femoral artery, left in place for 1 minute to denude and dilate the artery, then removed.

Measurement of Neointimal Hyperplasia

For morphometric studies, femoral arteries were harvested 4 weeks after injury, and digitalized images of these vessels were obtained and analyzed using image analysis software (Version 3.2; Soft Imaging System, Munster, Germany). The lumen, internal elastic lamina (IEL), and external elastic lamina (EEL) were defined, and the intimal (tissue between lumen and IEL) and medial (tissue between IEL and EEL) areas were recorded. Neointima/media area (NI/M) ratios were also calculated.

Immunohistochemistry and Immunofluorescent Staining

Methods for immunohistochemistry and immunofluorescent staining were performed standard methods, and their details were described online only. Please see http://atvb.ahajournals.org.

cDNA Array Analysis

Total RNA was isolated from pooled arteries (n=6 for each group) using a QIAGEN RNeasy Minikit (QIAGEN Inc, Valencia, Calif). Murine U74A version 2 GeneChips were purchased from Affymetrix (Santa Clara, Calif) and hybridization was carried out according to the manufacturer's instructions.

Measurement of Proinflammatory Cytokine mRNA

RNA was isolated from pooled arteries (n=6 to 8 for each group) using a QIAGEN RNeasy Minikit (QIAGEN Inc, Valencia, Calif) and then amplified using a MessageAmp™ Kit (Ambion, Austin, Tex), which enables amplification of very small amounts of RNA. RNA also was isolated from hearts using TRIzol Reagent (Invitrogen, Carlsbad, Calif), after which cDNA was generated using both RNA samples and an Invitrogen SuperScript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, Calif). Real-time polymerase chain reaction (PCR) was then performed in an ABI-Prism 7700 (Applied Biosystems, Foster City, Calif) using Taqman Universal PCR MasterMix (Applied Biosystems). The oligonucleotide probes and primers for IL-6, MCP-1, VEGF, transforming growth factor (TGF)-β,stromal cell-derived factor (SDF)-1α, IL-1β, and TNF-α were purchased from Applied Biosystems.

Measurements of Plasma TNF-α Levels

Plasma TNF-α levels were measured using a mouse TNF-α enzyme-linked immunosorbent assay kit (eBioscience, San Diego, Calif) according to the manufacturer instructions. The minimum detectable concentration of TNF-α was 8 pg/mL.

Experimental Protocols

Depending on the experiment, mice were placed into one of four groups: the AMI+VI group were subjected to both AMI and femoral arterial injury; the VI group was subjected to a sham operation and femoral arterial injury; and the AMI and sham-operated groups received only AMI or the sham operation, respectively. Mice that did not receive BM cells were used to compare neointimal hyperplasia and mRNA expression among the groups. Two weeks after AMI, femoral arteries were carefully excised from 6 to 8 mice in each group and pooled for analysis of mRNA expression. Data from 2 independent experiments were averaged. Four weeks after AMI, femoral arteries were excised from 10 mice in each group to measure neointimal hyperplasia. Again, 2 series of these experiments were performed. In addition, to detect BM-derived cells within the neointima, we performed similar experiments using 6 mice that had received BM cells in each group.

In some mice in AMI+VI and VI groups, PTX (30 mg/kg per day) or vehicle (Veh) (phosphate-buffered saline) was infused intraperitoneally using an osmotic minipump (Alzet, Cupertino, Calif) for 4 weeks after AMI or sham operation. At the end of the 4-week treatment period, the mice were euthanized and peripheral blood was collected to measure circulating TNF-α levels, and the injured and sham-operated femoral arteries were collected to assess the neointimal hyperplasia.

Statistical Analysis

All results are expressed as means±SEM. Differences between groups were evaluated for statistical significance using Student t test. Values of $P<0.05$ were considered significant.

Results

Myocardial Infarction Augments Neointimal Hyperplasia in Injured Arteries

Four weeks after the surgery, neointima formation was observed in mice in both the AMI+VI and VI groups (Figure 1A and 1B). As can be seen in Figure 1, however, the neointimal hyperplasia was substantially more prominent in

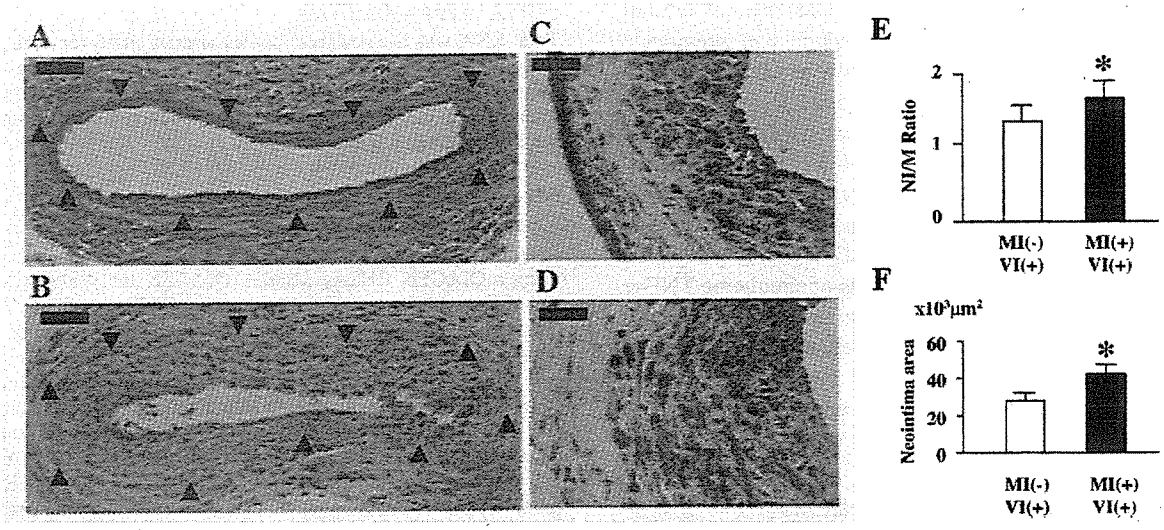

Figure 1. AMI augments neointimal hyperplasia in injured femoral arteries. A and B, Hematoxylin and eosin-stained sections of femoral artery from VI (A) and AMI+VI (B) mice (8 to 10 weeks old) harvested 4 weeks after vascular injury; scale bars represent 50 μm. C and D, α-SMA immunostained sections of femoral artery from VI (C) and AMI+VI (D) mice; scale bars represent 25 μm. Arrowheads indicate the internal elastic lamina. E,F, NI/M ratios (E) and neointimal area (F) in the injured femoral arteries of VI (open bars) and AMI+VI (solid bars) mice. Bars are means±SEM of 8 mice per group;*$P<0.05$ vs the VI group.

the AMI+VI group than in the VI group. Immunohistochemical staining revealed that the neointimas in both groups were mainly composed of α-SMA-positive SMCs (Figure 1C and 1D), suggesting that the inflammatory response to AMI increases SMC numbers within the neointimas of distant injured arteries. When we measured the neointimal and medial areas using computerized morphometry, we found that the NI/M ratios and neointimal areas were significantly greater in the AMI+VI group than in the VI group (Figure 1E and 1F).

BM-Derived Cells Contribute to Neointima Formation but Not to the AMI-Related Augmentation

To determine the extent to which BM-derived cells contribute to the AMI-related augmentation of neointimal hyperplasia in injured arteries, we next performed a set of experiments using mice that had received BM cells from GFP mice. Four weeks after the vascular injury, we observed that GFP-positive cells had accumulated in the neointimas and medias of the injured arteries (Figure 2A and 2B) in both AMI+VI and VI mice. Moreover, immunofluorescent staining showed that some of the GFP-positive cells expressed α-SMA (Figure 2C), suggesting they had differentiated into cells similar to SMCs. The numbers of GFP-positive cells did not significantly differ in the neointimas or medias of mice in the AMI+VI and VI groups (Figure 2D), though they tended to be larger in AMI+VI mice than in VI mice.

It thus appears that BM-derived cells do indeed contribute to vascular remodeling after injury, but they are not responsible for the AMI-related augmentation of the response. It also appears that the inflammatory response to AMI did not promote significant mobilization of progenitor cells with the potential to differentiate into SMCs.

Expression of Proinflammatory Cytokines in Injured Femoral Arteries

Given the absence of a significant contribution by BM-derived cells to the augmented neointimal hyperplasia seen in injured arteries after AMI, we next sought to identify any molecules that might trigger migration and proliferation of medial SMCs by analyzing the expression profiles of various mRNAs using cDNA arrays. Among a number of upregulated molecules, levels of IL-6, MCP-1, VEGF, TGF-β, SDF-1α, and IL-1βmRNA were markedly higher in the injured arteries of AMI+VI mice than in those of sham-operated mice. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed ≈7-fold increase in IL-6 mRNA expression in the AMI+VI group, as compared with the VI group, and ≈500-fold increase, as compared with the sham-operated group (Figure 3A). Levels of MCP-1, VEGF, TGF-β, SDF-1α and IL-1β mRNA were similar in both the AMI+VI and VI groups and higher than in the sham-operated group (supplemental Figure IA to IE, available online at http://atvb.ahajournals.org.). In addition, immunohistochemical analysis showed clear upregulation of IL-6 protein that paralleled the upregulation of mRNA expression in the neointimal region (Figure 3B).

Cardiac Expression of TNF-α After AMI

Because TNF-α reportedly stimulates IL-6 expression,[22] we next used quantitative RT-PCR to examine expression of TNF-α mRNA in infarcted hearts in an effort to determine the reason why IL-6 mRNA was preferentially upregulated in injured arteries following AMI. As shown in supplemental Figure IIA, expression of TNF-α mRNA was significantly increased in infarcted hearts 1, 3, 7, and 28 days after AMI, as compared with sham-operated hearts. Moreover, immunohistochemical analysis showed clear upregulation of TNF-α protein that paralleled the upregulation of mRNA expression in the infarcted hearts (supplemental Figure IIB and IIC).

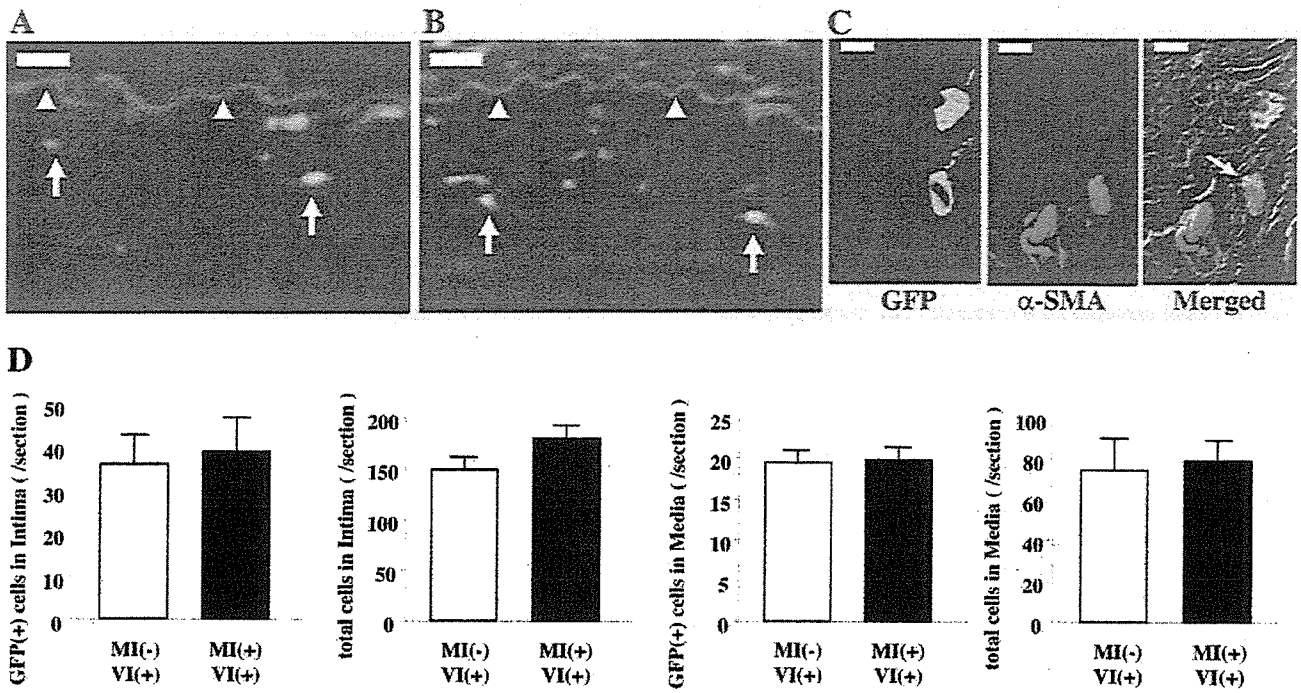

Figure 2. BM-derived GFP-positive cells within injured arteries. Vascular injuries were induced in $BMT^{GFP \rightarrow Wild}$ mice, after which the femoral arteries were fixed in 4% paraformaldehyde and embedded in plastic resin. Injured arteries from VI (A) and AMI+VI (B) mice were harvested after 4 weeks and observed under a confocal microscope. Arrowheads indicate the internal elastic lamina; arrows indicate GFP-positive cells; scale bars represent 25 μm. C, Immunofluorescent staining with Cy3-conjugated anti-α-SMA antibody (red) within injured arteries. The arrow indicates a GFP-positive SMC; scale bars represent 5 μm. D, Numbers of GFP-positive cells and total cell numbers within the injured arteries of VI (open bars) and AMI+VI (solid bars) mice. Bars are means±SEM for 5 mice per group.

Femoral Arterial Expression of TNFR1 After Vascular Injury

Given the increased cardiac expression of TNF-α and circulating of TNF-α levels after AMI, we tested the possibility that TNF-α acts via locally expressed TNFR1 to upregulate expression of IL-6 within injured arteries. Consistent with that idea, quantitative RT-PCR analysis revealed that the level of TNFR1 mRNA expression was significantly higher in injured femoral arteries from both AMI+VI and VI mice than in those from sham-operated or AMI mice (Figure 4).

Effect of Blockade of TNF-α Production on Vascular Remodeling

Finally, to confirm that the relationship between the increase in plasma TNF-α levels and the augmentation in neointima formation in remote injured arteries was causative, we tested the effects of PTX, an inhibitor of TNF-α synthesis. We found that plasma TNF-α levels were significantly higher in vehicle (Veh)-treated AMI+VI mice than in Veh-treated VI mice, but that TNF-α levels in AMI+VI mice were significantly diminished by PTX to a level similar to that seen in Veh-treated VI mice (Figure 5B). In addition, morphometric analysis revealed that neointimal areas and NI/M ratios in Veh-treated AMI+VI mice were significantly greater than in Veh-treated VI mice and that PTX significantly reduced neointimal areas and NI/M ratios (Figure 5A, 5C, and 5E). However, PTX treatment did not significantly affect neointimal areas or NI/M ratios in VI mice. Thus, prevention of the AMI-induced increase in plasma TNF-α levels by PTX attenuated neointimal hyperplasia in a remote artery after AMI.

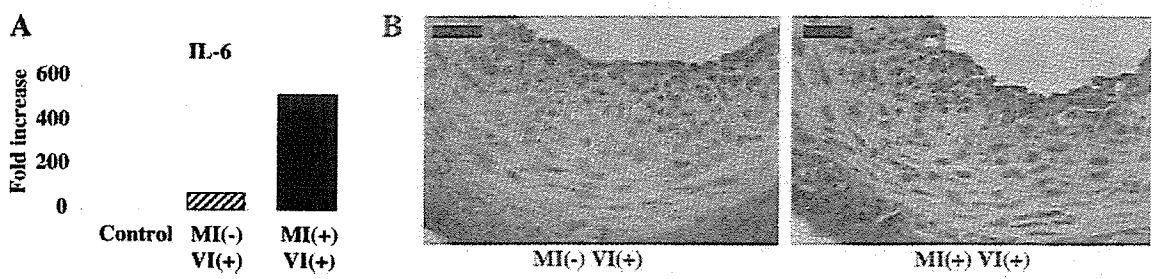

Figure 3. A, Effect of myocardial infarction on expression of the IL-6 within injured arteries: white bars, control; hatched bars, VI; black bar, AMI+VI. Tissue samples were prepared from injured and uninjured arteries 14 days after surgery (control). The result shown is representative of data obtained from 3 to 4 mice per group. IL-6 signal intensities were normalized to that of GAPDH; bars depict the fold increase relative to uninjured arteries (control). B, Immunohistochemical staining of IL6 within injured femoral arteries from VI and AMI+VI mice harvested 4 weeks after surgery. Scale bars represent 25 μm.

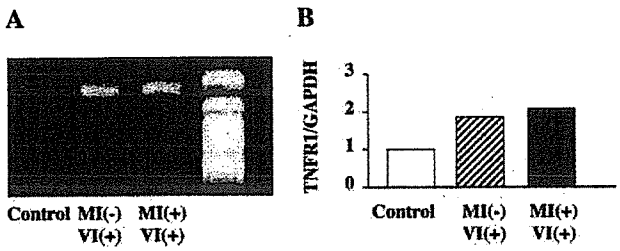

Figure 4. Effect of vascular injury on arterial expression of TNFR1. Tissue samples were prepared from injured and uninjured (control) femoral arteries 14 days after surgery: A, A representative image of RT-PCRs for TNFR1 expression. B, Quantitative real-time PCR analysis of TNFR1 expression levels obtained from 3 to 4 mice in each group.

Discussion

The main findings of the present study are that: (1) the inflammatory response to AMI augments neointimal hyperplasia at sites of injury within distant arteries; (2) BM-derived cells contribute to neointimal hyperplasia after vascular injury, but not to the AMI-related augmentation of the response; (3) cardiac synthesis of TNF-α and circulating TNF-α levels are both increased after AMI, as is expression of TNFR1 mRNA in injured arteries; (4) IL-6 is preferentially upregulated in the neointima of injured arteries after AMI; and (5) treatment with PTX, an inhibitor of TNF-α synthesis, inhibited the AMI-induced increases in plasma TNF-α and attenuated neointima formation after vascular injury. It thus appears that the inflammatory response to AMI stimulates neointimal hyperplasia at sites of vascular injury at least in part by stimulating signaling via TNF-α, TNFR1, and IL-6.

Cardiac levels of several vasoactive cytokines are elevated after AMI. For instance, levels of VEGF are increased in both infarcted hearts and the plasma. We also have observed that plasma levels of placental growth factor (PlGF), another VEGF family cytokine, are elevated within infarcted hearts as a result of its synthesis in endothelial cells within the infarcted region.[23] Earlier works by Hattori et al indicate that both VEGF and PlGF stimulate matrix metalloproteinase (MMP)-9 expression in BM stromal cells via the flt pathway, and that MMP-9 cleaves membrane bound Kit ligand into soluble Kit ligand, which in turn activates hematopoietic stem cells.[24,25] In addition, numbers of CD34-positive cells also are increased after AMI.[13] Based on these findings, we suggested that conditions directly caused by AMI stimulate neointimal hyperplasia at remote sites of vascular injury, and that BM-derived cells contribute significantly to the AMI-related augmentation of neointimal hyperplasia. In that regard, one recent report showed that BM-derived cells contribute to neointimal hyperplasia after mechanical vascular injury, especially after severe wire-induced injury.[26] In the present

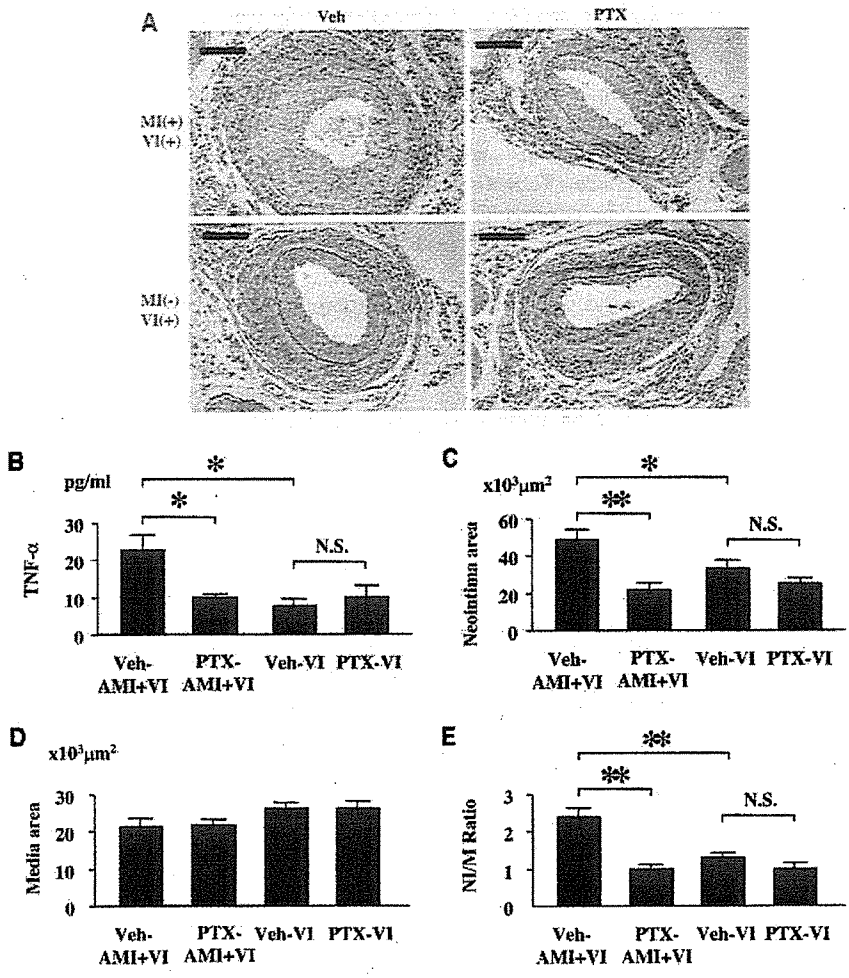

Figure 5. Inhibitory effect of PTX in AMI+VI and VI mice. A, Hematoxylin and eosin-stained sections of femoral artery from Veh-treated AMI+VI, Veh-treated VI, PTX-treated AMI+VI, and PTX-treated VI mice (8 to 10 weeks old) harvested 4 weeks after vascular injury; scale bars represent 100 μm. B, Circulating TNF-α levels in AMI+VI and VI mice continuously infused for 4 weeks with Veh or PTX. C to E, Effect of PTX treatment on morphological changes. Morphometric analysis of injured femoral arteries in AMI+VI and VI mice, with or without PTX, 4 weeks after wire-induced injury. Values are mean±SEM; *$P<0.05$; **$P<0.01$; n=6 to 10; N.S indicates not statistically significant.

study, we confirmed the presence of BM-derived cells in the neointima and media of the affected artery after wire-induced vascular injury. But as shown in Figure 2, although the number of neointimal BM-derived cells tended to be higher in mice after AMI than after sham operation, the difference was not sufficient to explain the augmented neointimal hyperplasia seen after AMI. Apparently, AMI does not stimulate recruitment of BM-derived cells into the neointima at sites of vascular injury. Pathological significance of BM-derived cells in the setting of AMI is needed to be further elucidated.

Our analysis of cDNA arrays, performed to identify key molecules responsible for the AMI-related augmentation of neointimal hyperplasia after vascular injury, revealed upregulation of MCP-1, VEGF, TGF-β, SDF-1α, and IL-1β within injured arteries, with and without AMI. However, levels of IL-6 were $\approx$7-fold higher in the injured arteries of AMI+VI mice than in those of VI mice. Studies have shown that IL-6 mRNA is expressed in the atherosclerotic lesions of apolipoprotein E knockout mice[27] and humans[28,29] and in the neointimas of injured arteries,[5] and that STAT3, which is activated by IL-6, contributes to neointima formation by promoting neointimal SMC proliferation and survival.[30] However, earlier reports mainly emphasized the importance of MCP-1, VEGF, TGF-β,SDF-1α, and IL-1β rather than IL-6 in neointima formation. The present study confirms the importance of IL-6 in neointimal hyperplasia after wire-induced injury and suggests that IL-6 plays a more important role in AMI-related neointimal hyperplasia than the other aforementioned mediators.

Bearing that in mind, a key question is, what are the signals that stimulate the preferential elevation of IL-6 expression in injured arteries after AMI? A number of earlier reports have shown that TNF-α and IL-1β are strong stimulators of IL-6 expression.[22,31] Consistent with that earlier work, we found that TNF-α expression is upregulated in infarcted hearts during the 2-week period after AMI and that plasma TNF-α levels were increased to 22.6$\pm$4.2 pg/mL, which is sufficient to stimulate IL-6 expression.[32–34] We also detected expression of TNFR1 mRNA in injured arteries, but not in healthy ones. TNF-α exerts its effects through both TNFR1 and TNFR2, but blockade of TNFR1 gene expression reportedly reduces neointimal hyperplasia after vascular injury by 2-fold, whereas blocking TNFR2 expression has no effect.[35] Thus, in the setting of AMI, it is likely that TNF-α released from the infarcted heart binds to newly upregulated-TNFR1 on the surface of cells at remote sites of arterial injury, leading to IL-6 production and, ultimately, stimulation of neointimal hyperplasia.

To prove a cause–effect relationship between expression of TNF-α in heart and IL-6 in arteries, and one between AMI and augmented neointima formation, we demonstrated that prevention of TNF-α synthesis by PTX inhibited the AMI-induced increases in plasma TNF-α and attenuated neointimal formation after vascular injury. Collectively, these results are indicative of the important role played by TNF-α in experimental postangioplasty restenosis. An earlier report showed the short-term, exogenous administration of TNF-α did not increase neointima formation after balloon injury in rabbits,[36] suggesting that prolonged and persistent elevation of circulating TNF-α is required to promote neointimal hyperplasia after vascular injury. In this study, we used PTX as an inhibitor of TNF-α synthesis instead of a neutralizing antibody or a soluble form of TNF-α receptor. Although both neutralizing antibody and soluble form of TNF-αreceptor are more specific than PTX, they are immunogenic,[37] when they are used in vivo experiments especially in the long-term experiment like the present experiment. PTX reduces the synthesis of TNF-α by blocking its transcription,[38] and has been used successfully in earlier works.[39,40] Therefore, we adopted PTX to inhibit TNF-α pathway. However, it might be possible that present findings were modulated by other molecules transcriptionally suppressed by PTX.

In the clinical point of view, primary purpose of the present study is to elucidate whether post-AMI conditions enhance the neointimal hyperplasia and restenosis after PCI, that is why we generated a mouse model of AMI plus vascular injury. However, further studies using model in larger animal such as porcine are necessary to get more directly evidence that AMI augments neointimal hyperplasia and restenosis after PCI of infarct-related artery.

In conclusion, the present findings provide experimental evidence supporting the idea that conditions directly resulting from AMI exacerbate neointimal hyperplasia after vascular injury through activation of TNF-α, TNFR1, and IL-6 network.

Acknowledgments

We thank Professor M. Okabe, Osaka University, Osaka, Japan, for providing us with GFP Tg mice.

Sources of Funding

This work was supported in part by research grants from the Japanese Ministry of Health, Labor and Welfare, the Japanese Society for the Promotion of Science, and the Japan Health Science Foundation, and a Japan Heart Foundation/Pfizer Japan Grant on Cardiovascular Disease Research.

Disclosures

None.

References

1. Hansson GK. Immune mechanisms in atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2001;21:1876–1890.
2. Angioi M, Abdelmouttaleb I, Rodriguez RM, Aimone-Gastin I, Adjalla C, Guéant JL, Danchin N. Increased C-reactive protein levels in patients with in-stent restenosis and its implications. *Am J Cardiol.* 2001;87:1189–1193.
3. Farb A, Sangiorgi G, Carter AJ, Walley VM, Edwards WD, Schwartz RS, Virmani R. Pathology of acute and chronic coronary stenting in humans. *Circulation.* 1999;99:44–52.
4. Serrano CV Jr., Ramires JA, Venturinelli M, Arie S, D'Amico E, Zweier JL, Pileggi F, da Luz PL. Coronary angioplasty results in leukocyte and platelet activation with adhesion molecule expression. Evidence of inflammatory responses in coronary angioplasty. *J Am Coll Cardiol.* 1997;29:1276–1283.
5. Wu L, Iwai M, Nakagami H, Li Z, Chen R, Suzuki J, Akishita M, Gasparo MD, Horiuchi M. Roles of angiotensin II type 2 receptor stimulation associated with selective angiotensin II type 1 receptor blockade with Valsartan in the improvement of inflammation-induced vascular injury. *Circulation.* 2001;104:2716–2721.
6. Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. *Cardiovasc Res.* 2002;53:31–47.
7. Irwin MW, Mak S, Mann DL, Qu R, Penninger JM, Yan A, Dawood F, Wen WH, Shou Z, Liu P. Tissue expression and immunolocalization of

tumor necrosis factor-α in postinfarction dysfunctional myocardium. *Circulation.* 1999;99:1492–1498.
8. Kukielka GL, Smith CW, Manning AM, Youker KA, Michael LH, Entman ML. Induction of interleukin-6 synthesis in the myocardium. Potential role in post-reperfusion inflammatory injury. *Circulation.* 1995; 92:1866–1875.
9. Lee SH, Wolf PL, Escudero R, Deutsch R, Jamieson SW, Thistlethwaite PA. Early expression of angiogenesis factors in acute myocardial ischemia and infarction. *N Engl J Med.* 2000;342:626–633.
10. Li J, Brown LF, Hibberd MG, Grossman JD, Morgan JP, Simons M. VEGF, flk-1, and flt-1 expression in a rat myocardial infarction model of angiogenesis. *Am J Physiol.* 1996;270:H1803–H1811.
11. Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T, Hirai H, Makuuchi M, Hirata Y, Nagai R. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med.* 2002;8:403–409.
12. Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med.* 1999; 340:115–126.
13. Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki K, Shimada T, Oike Y, and Imaizumi T. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation.* 2001;103:2776–2779.
14. Massa M, Rosti V, Ferrario M, Campanelli R, Ramajoli I, Rosso R, De Ferrari GM, Ferlini M, Goffredo L, Bertoletti A, Klersy C, Pecci A, Moratti R, Tavazzi L. Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction. *Blood.* 2005;105:199–206.
15. Francis J, Chu Y, Johnson AK, Weiss RM, Felder RB. Acute myocardial infarction induces hypothalamic cytokine synthesis. *Am J Physiol Heart Circ Physiol.* 2004;286:H2264–H2271.
16. Waage A, Sorensen M, Stordal B. Differential effect of oxpentifylline on tumour necrosis factor and interleukin-6 production. *Lancet.* 1990; 335:543.
17. Zabel P, Wolter DT, Schonharting MM, Schade UF. Oxpentifylline in endotoxaemia. *Lancet.* 1989;334:1474–1477.
18. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. Green mice as a source of ubiquitous green cells. *FEBS Lett.* 1997;407:313–319.
19. Adachi Y, Saito Y, Kishimoto I, Harada M, Kuwahara K, Takahashi N, Kawakami R, Nakanishi M, Nakagawa Y, Tanimoto K, Saitoh Y, M, Yasuno S, Usami S, Iwai M, Horiuchi M, Nakao K. Angiotensin II type 2 receptor deficiency exacerbates heart failure and reduces survival after acute myocardial infarction in mice. *Circulation.* 2003;107:2406–2408.
20. Izumi T, Saito Y, Kishimoto I, Harada M, Kuwahara K, Hamanaka I, Takahashi N, Kawakami R, Li Y, Takemura G, Fujiwara H, Garbers DL, Mochizuki S, Nakao K. Blockade of the natriuretic peptide receptor guanylyl cyclase-A inhibits NF-κB activation and alleviates myocardial ischemia/reperfusion injury. *J Clin Invest.* 2001;108:203–213.
21. Sata M, Maejima Y, Adachi F, Fukino K, Saiura A, Sugiura S, Aoyagi T, Imai Y, Kurihara H, Kimura K, Omata M, Makuuchi M, Hirata Y, Nagai R. A mouse model of vascular injury that induces rapid onset of medial cell apoptosis followed by reproducible neointimal hyperplasia. *J Mol Cell Cardiol.* 2000;32:2097–2104.
22. Vanden Berghe W, Vermeulen L, De Wilde G, De Bosscher K, Boone E, Haegeman G. Signal transduction by tumor necrosis factor and gene regulation of the inflammatory cytokine interleukin-6. *Biochem Pharmacol.* 2000;60:1185–1195.
23. Iwama H, Uemura S, Naya N, Imagawa K, Takemoto Y, Asai O, Onoue K, Okayama S, Somekawa S, Kida Y, Takeda Y, Takaoka M, Kawata H, Horii M, Nakajima T, Doi N, Saito Y. Cardiac expression of placental growth factor predicts the improvement of chronic phase left ventricular function in patients with acute myocardial infarction. *J Am Coll Cardiol.* 2006;47:1559–1567.
24. Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, Hicklin DJ, Zhu Z, Bohlen P, Witte L, Hendrikx J, Hackett NR, Crystal RG, Moore MA, Werb Z, Lyden D, Rafii S. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat Med.* 2002;8:841–849.
25. Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, Crystal RG, Besmer P, Lyden D, Moore MA, Werb Z, Rafii S. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell.* 2002;109:625–637.
26. Tanaka K, Sata M, Hirata Y, Nagai R. Diverse contribution of bone marrow cells to neointimal hyperplasia after mechanical vascular injuries. *Circ Res.* 2003;93:783–790.
27. Sukovich DA, Kauser K, Shirley FD, DelVecchio V, Halks-Miller M, Rubanyi GM. Expression of interleukin-6 in atherosclerotic lesions of male ApoE-knockout mice: inhibition by 17beta-estradiol. *Arterioscler Thromb Vasc Biol.* 1998;18:1498–1505.
28. Rus HG, Vlaicu R, Niculescu F. Interleukin-6 and interleukin-8 protein and gene expression in human arterial atherosclerotic wall. *Atherosclerosis.* 1996;127:263–271.
29. Seino Y, Ikeda U, Ikeda M, Yamamoto K, Misawa Y, Hasegawa T, Kano S, Shimada K. Interleukin 6 gene transcripts are expressed in human atherosclerotic lesions. *Cytokine.* 1994;6:87–91.
30. Shibata R, Kai H, Seki Y, Kato S, Wada Y, Hanakawa Y, Hashimoto K, Yoshimura A, Imaizumi T. Inhibition of STAT3 prevents neointima formation by inhibiting proliferation and promoting apoptosis of neointimal smooth muscle cells. *Hum Gene Ther.* 2003;14:601–610.
31. Loppnow H, Libby P. Proliferating or interleukin 1-activated human vascular smooth muscle cells secrete copious interleukin 6. *J Clin Invest.* 1990;85:731–738.
32. Francis J, Zhang ZH, Weiss RM, Felder RB. Neural regulation of the proinflammatory cytokine response to acute myocardial infarction. *Am J Physiol Heart Circ Physiol.* 2004;287:H791–H797.
33. Selzman CH, Shames BD, Reznikov LL, Miller SA, Meng X, Barton HA, Werman A, Harken AH, Dinarello CA, Banerjee A. Liposomal delivery of purified inhibitory-κBa inhibits tumor necrosis factor-α–induced human vascular smooth muscle proliferation. *Circ Res.* 1999;84: 867–875.
34. Ridker PM, Rifai N, Pfeffer M, Sacks F, Lepage S, Braunwald E. for the Cholesterol And Recurrent Events (CARE) Investigators. Elevation of tumor necrosis factor-α and increased risk of recurrent coronary events after myocardial infarction. *Circulation.* 2000;101:2149–2153.
35. Zimmerman MA, Reznikov LL, Sorensen AC, Selzman CH. Relative contribution of the TNF-alpha receptors to murine intimal hyperplasia. *Am J Physiol Regul Integr Comp Physiol.* 2003;284:R1213–1218.
36. Miller AM, McPhaden AR, Preston A, Wadsworth RM, Wainwright CL. TNFalpha increases the inflammatory response to vascular balloon injury without accelerating neointimal formation. *Atherosclerosis.* 2005;179: 51–59.
37. Douglas L. Mann. Inflammatory mediators and the failing heart. Past, present, and the foreseeable future. *Circ Res.* 2002;91:988–998.
38. Strieter RM, Remick DG, Ward PA, Spengler RN, Lynch JP 3rd, Larrick J, Kunkel SL. Cellular and molecular regulation of tumor necrosis factor-alpha production by pentoxifylline. *Biochem Biophys Res Commun.* 1988;155:1230–1236.
39. Sliwa K, Skudicky D, Candy G, Wisenbaugh T, Sareli P. Randomised investigation of effects of pentoxifylline on left ventricular performance in idiopathic dilated cardiomyopathy. *Lancet.* 1998;351:1091–1093.
40. Sliwa K, Woodiwiss A, Kone VN, Candy G, Badenhorst D, Norton G, Zambakides C, Peters F, Essop R. Therapy of ischemic cardiomyopathy with the immunomodulating agent pentoxifylline: results of a randomized study. *Circulation.* 2004;109:750–755.

Available online at www.sciencedirect.com

SCIENCE DIRECT

Metabolism Clinical and Experimental 55 (2006) 300–308

Metabolism
Clinical and Experimental

www.elsevier.com/locate/metabol

α2 Isoform–specific activation of 5′ adenosine monophosphate–activated protein kinase by 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside at a physiological level activates glucose transport and increases glucose transporter 4 in mouse skeletal muscle

Masako Nakano[a], Taku Hamada[a], Tatsuya Hayashi[a,b,*], Shin Yonemitsu[a], Licht Miyamoto[a], Taro Toyoda[c], Satsuki Tanaka[a], Hiroaki Masuzaki[a], Ken Ebihara[a], Yoshihiro Ogawa[a], Kiminori Hosoda[a], Gen Inoue[a], Yasunao Yoshimasa[a], Akira Otaka[d], Toru Fushiki[c], Kazuwa Nakao[a]

[a]*Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan*
[b]*Kyoto University Graduate School of Human and Environmental Studies, Kyoto 606-8501, Japan*
[c]*Division of Food Science and Biotechnology, Kyoto University Graduate School of Agriculture, Kyoto 606-8502, Japan*
[d]*Department of Bioorganic Medicinal Chemistry, Kyoto University Graduate School of Pharmaceutical Sciences, Kyoto 606-8501, Japan*

Received 27 October 2004; accepted 24 September 2005

Abstract

5′ Adenosine monophosphate–activated protein kinase (AMPK) has been implicated in exercise-induced stimulation of glucose metabolism in skeletal muscle. Although skeletal muscle expresses both the α1 and α2 isoforms of AMPK, the α2 isoform is activated predominantly in response to moderate-intensity endurance exercise in human and animal muscles. The purpose of this study was to determine whether activation of α2 AMPK plays a role in increasing the rate of glucose transport, promoting glucose transporter 4 (GLUT4) expression, and enhancing insulin sensitivity in skeletal muscle. To selectively activate the α2 isoform, we used 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR), which is metabolized in muscle cells and preferentially stimulates the α2 isoform. Subcutaneous administration of 250 mg/kg AICAR activated the α2 isoform for 90 minutes, but not the α1 isoform in hind limb muscles of the C57/B6J mouse. The maximal activation of the α2 isoform was observed 30 to 60 minutes after administration of AICAR and was similar to the activation induced by a 30-minute swim in a current pool. The increase in α2 activity paralleled the phosphorylation of Thr^{172}, the essential residue for full kinase activation, and the activity of acetyl-coenzyme A carboxylase β, a known substrate of AMPK in skeletal muscle. Subcutaneous injection of AICAR rapidly increased, by 30%, the rate of 2-deoxyglucose (2DG) transport into soleus muscle; 2DG transport increased within 30 minutes and remained elevated for 4 hours after administration of AICAR. Repeated intraperitoneal injection of AICAR, 3 times a day for 4 to 7 days, increased soleus GLUT4 protein by 30% concomitant with a significant 20% increase in insulin-stimulated 2DG transport. These data suggest that moderate endurance exercise promotes glucose transport, GLUT4 expression, and insulin sensitivity in skeletal muscle at least partially via activation of the α2 isoform of AMPK.

1. Introduction

Physical exercise is a potent stimulator of glucose transport and glucose transporter 4 (GLUT4) expression in skeletal muscle. An acute bout of exercise increases the rate of glucose transport into contracting muscles by inducing translocation of GLUT4 to the cell surface via an insulin-independent mechanism (contraction-stimulated glucose transport) [1]. Acute exercise also activates expression of GLUT4 protein, and the GLUT4 protein expression is elevated with repeated bouts of acute exercise [2]. The exercise-induced increase in GLUT4 is associated with improved insulin sensitivity (ie, increased rates of insulin-stimulated GLUT4 translocation and glucose transport into

* Corresponding author. Kyoto University Graduate School of Human and Environmental Studies, Kyoto 606-8501, Japan. Tel.: +81 75 753 6640; fax: +81 75 753 6640.
E-mail address: tatsuya@kuhp.kyoto-u.ac.jp (T. Hayashi).

0026-0495/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2005.09.003