Mini Review

The anti-inflammatory and vasculo-neuro-regenerative roles of adrenomedullin in ischemic brain

Kazutoshi Miyashita¹,², *, Hiroshi Itoh¹,², and Kazuwa Nakao²

¹Keio University, Department of Internal Medicine, Tokyo, Japan
²Kyoto University Graduate School of Medicine, Department of Medicine and Clinical Science, Kyoto, Japan

Adrenomedullin (AM) is a vasodilating peptide secreted mainly from vascular wall, and its expression is markedly enhanced after stroke. We developed novel AM-transgenic (AM-Tg) mice and performed middle cerebral artery occlusion for 20 min (20m-MCAO) to examine the effects of AM on degenerative and regenerative processes in ischemic brain. The infarct area and gliosis after 20m-MCAO was reduced in AM-Tg mice in association with suppression of leukocyte infiltration, oxidative stress, and apoptosis in the ischemic core. In addition, vascular regeneration and subsequent neurogenesis were enhanced in AM-Tg mice, preceded by increase in mobilization of CD34+ cells, which can differentiate into endothelial cells. The vasculo-neuro-regenerative actions observed in AM-Tg mice in combination with anti-inflammatory and neuro-protective effects resulted in improved recovery of motor function. In vitro, AM exerted direct anti-apoptotic and neurogenic actions on neuronal cells. In summary, this study provides a basis for new strategy to rescue ischemic brain by AM through its multiple hormonal actions which concurrently lead to anti-inflammation, neuro-protection and vasculo-neuro-regeneration.


* Correspondence should be addressed to:
Kazutoshi Miyashita, Keio University, Department of Internal Medicine, Reserch Park 5N8, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, Japan. Tel: +81-3-5363-3878, email: miyakaz@sc.ite.keio.ac.jp

Key words adrenomedullin, anti-inflammation, neuro-protection, angiogenesis, neurogenesis, cerebral ischemia, stroke

Adrenomedullin (AM) is a potent vasodilating peptide which is secreted mainly from the vascular wall into circulating blood¹. It reduces pre- and after-load on the heart and intravenous administration of AM to patients with heart failure has already been revealed beneficial hemodynamic effects². Along with its vasodilating action, a number of studies have demonstrated various and significant effects of AM on the regulation of vascular structure, including its development, remodeling, and regeneration. Mice lacking the AM gene did not survive their embryonic stage and showed abnormal vasculature with subcutaneous hemorrhage³,⁴. Mice over-expressing AM in endothelial cells were revealed to be hypotensive and resistant to vascular remodeling such as neointima formation caused by cuff injury⁵. We have established that AM promotes endothelial regeneration in an assay using cultured endothelial cells and enhances neo-vascularization into subcutaneously implanted gel-plugs in mice⁶,⁷. We and others have further demonstrated that the potentiating action of AM on vascular regeneration is mediated by activa-
Fig. 1 Effects of AM on infarct area and gliosis after 20m-MCAO

(A and B) Representative images of the ischemic striatum on postoperative day 7 stained for NeuN (blue) and GFAP (green) in Wt and AM-Tg mice. (A) Infarct area, defined as the region where NeuN immuno-reactivity was lost. (B) Gliosis, defined as the area where GFAP immuno-reactivity was observed. Scale bar, 500 μm (x5 magnification) (C and D) Quantitative analysis of (C) the infarct area and (D) gliosis. *p < 0.05; ns, not significant for Wt vs. AM-Tg; n = 12. (E and F) Effects of AM on leukocyte infiltration, ROS production, and apoptosis in the ischemic brain after 20m-MCAO in Wt and AM-Tg. (E) Detection of leukocyte infiltration in the ischemic core on postoperative day 7 by immuno-staining for CD45+ cells (red). Arrows, CD45+ cells. (F) Detection of apoptotic cells in the ischemic core on postoperative day 7 by immuno-staining for ssDNA+ cells (green). Arrows, ssDNA+ cells. (G and H) Quantitative analysis of (G) CD45+ cells and (H) ssDNA+ cells in the ischemic core. *p < 0.05; **p < 0.01; ns, not significant for Wt vs. AM-Tg; n=12. Scale bar, 100 μm (x20 magnification).

The figure was cited from Reference 10 (Miyashita K et al. Endocrinology 147:1642-1653; DOI: 10.1210/en.2005-1038) with modification.

Fig. 2 Effects of AM on vascular regeneration in the ischemic brain after 20m-MCAO

(A-D) Analysis of the blood flow in the ischemic brain by LDPI evaluated in (A) mice with the scalp removed. Flowmetric analysis of the ischemic brain (B) during MCA-Occlusion and (C and D) on day 28 after 20m-MCAO in (C) Wt and (D) AM-Tg mice. Red or white indicates higher flow than blue or green. (E-G) Histological examination of the vasculature in the ischemic core with PECAM-1 staining. Ischemic striatum on day 28 after 20m-MCAO in (E) Wt and (F) AM-Tg, and (G) contralateral nonischemic striatum. Scale bar, 100 μm (x20 magnification). (H) Quantitative analysis of the blood flow in the ischemic brain. Comparison of recovery from ischemia after 20m-MCAO between Wt and AM-Tg. MCA-Oc, blood flow during MCA occlusion; **p < 0.01 for Wt vs. AM-Tg by ANCOVA; n=8. (I) Quantitative analysis of capillary density in the ischemic brain. Comparison of time course for increase in capillary density, determined as the number of PECAM-1+ cells, between Wt and AM-Tg mice. *p < 0.05; ns, not significant; n=8. (J and K) Effects of AM on neurogenesis and recovery of impaired motor function after 20m-MCAO. (J) Quantitative analysis of regenerated neurons. *p < 0.05; ns, not significant; n=12. (K) Recovery of impaired motor function after 20m-MCAO, quantified as the exercise time on an accelerating rotorod from the start to fall down. *p < 0.05; **p < 0.01 for Wt vs. AM-Tg by ANCOVA; n=14. The figure was cited from Reference 10 (Miyashita K et al. Endocrinology 147:1642-1653; DOI: 10.1210/en.2005-1038) with modification.
tion of the phosphatidyl inositol-3 kinase (PI3K)-Akt dependent pathway\(^6\).

It has been demonstrated that AM expression is markedly enhanced by ischemia through the activation of hypoxia-responsive elements in the AM gene via transcription factor hypoxia-inducible factor-1. In the central nervous system, where AM is mainly expressed in neurons and the endothelium, it is reported that transient ischemia boosted AM expression for more than 15 days\(^6\). However, the role of augmented AM in ischemic brain has remained unclear so far. In this context, we focused on the roles of AM in the ischemic brain subjected to 20-min middle cerebral artery occlusion (20m-MCAO) and examined its therapeutically potential using genetically engineered mice model.

We generated new lines of transgenic mice that overproduce AM (AM-Tg) in the liver that mimics chronic AM administration and induced 20m-MCAO to produce a nonfatal stroke model\(^10\). We observed the long-term effects of AM on the ischemic brain up to postoperative day 56 and immuno-histochemically examined the ischemic striatum to determine effects of AM on neuronal loss/apoptosis, gliosis, leukocyte infiltration, oxidative stress, vascular regeneration, and neurogenesis after 20m-MCAO. The plasma concentration of AM in the Tg mice was 585.5 ± 117.7 fmol/ml and the value was approximately 100 times more than endogenous AM. There were no apparent differences between genotypes in overall appearance, behavior and anatomical findings in the central nervous system. The systolic blood pressure (BP) in 12-wk-old mice was significantly reduced in the AM-Tg mice compared with Wt; the BP (mmHg) was 122.7 ± 1.6 in Wt vs. 109.4 ± 2.5 in the Tg mice. 20m-MCAO caused selective loss of NeuN-positive cells (neurons) and marked reactive gliosis, quantified as the number of glial fibrillary acidic protein (GFAP) positive cells, in the ipsi-lateral striatum within 24 hours after the operation; this condition was different from pan-necrosis caused by longer MCAO (e.g. 2h-MCAO in rodents). The infarct area, that is, the area of neuronal loss, expanded progressively up to day 7, and then showed gradual increase in size until day 56, whereas gliosis spread in parallel.

In AM-Tg mice, the infarct area and gliosis were reduced after 20m-MCAO along with suppression of leukocyte infiltration and ROS production\(^10\). A significant decrease in infarct area and gliosis was observed in AM-Tg mice after postoperative day 7, which was not obvious on day 3 (Fig.1A-D). Leukocyte infiltration quantified as the number of CD45+ cells was significantly suppressed in AM-Tg mice especially during day 3 to 7. In situ ROS production detected by staining with dihydroethidium was enhanced in Wt compared to that in AM-Tg mice. As a result, apoptotic cells quantified as the number of single-strand DNA (ssDNA)+ cells in the ischemic core were significantly reduced in the AM-Tg mice (Fig.1E-H).

Enhanced vascular regeneration in the striatum and augmented neurogenesis were also observed in AM-Tg mice after 20m-MCAO\(^10\). The blood flow in the ischemic brain estimated by a laser Doppler perfusion imager (LDPI) was significantly higher in AM-Tg mice after postoperative day 7 and higher flow was maintained until day 56. We confirmed that capillary density determined as the number of platelet endothelial cell adhesion molecule (PECAM)-1+ vascular endothelial cell was augmented in AM-Tg mice (Fig.2A-I). Peripheral CD34+ mononuclear cells, which are known to differentiate into endothelial cells and contribute to vasculogenesis, underwent physiological enhancement after 20m-MCAO; and further increase in AM-Tg mice was observed on day 3 to 7. Thus, the physiological neo-vascularization in the ischemic core after stroke was augmented in AM-Tg mice.

BrdU injection on postoperative day 4 to 6 proved that most BrdU-positive cells were co-stained with a glial marker, GFAP and that there were far fewer BrdU-PECAM-1 or BrdU-NeuN double-positive cells. We found that regenerated neurons defined as BrdU-NeuN double-positive cells were frequently detected adjacent to the vasculature and the number of these cells on day 56 was correlated with capillary density (Fig.2J). The cells increased from postoperative day 7 to 56 and their number was significantly higher in AM-Tg mice. Recovery of impaired motor function after 20m-MCAO, quantified as the exercise time on an accelerating rota-rod from the start to collapse down, was significantly better in AM-Tg mice (Fig.2K). To confirm whether vasculogenesis and neurogenesis are the contributing factor to

---

**Table 1** Significant correlation between the regenerative elements and apoptosis, neurogenesis, and functional recovery after 20m-MCAO

<table>
<thead>
<tr>
<th>X</th>
<th>Y</th>
<th>Regression line</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary density%</td>
<td>Apoptotic cell/mm³</td>
<td>Y=2.3X+97</td>
<td>0.01</td>
</tr>
<tr>
<td>Capillary density%</td>
<td>Regenerated neuron/mm³</td>
<td>Y=0.3X+13</td>
<td>0.008</td>
</tr>
<tr>
<td>Capillary density%</td>
<td>Regenerated neuron/mm³</td>
<td>Y=1.0X+0</td>
<td>0.096</td>
</tr>
</tbody>
</table>

The table was cited from Reference 10 Miyoshita K et al. Endocrinology 147:1642-1653. DOI: 10.1210/en.2005-1030 with modification.
the recovery from the ischemic damage, we analyzed the relation between capillary density, the number of regenerated neuron and the rota-rod result in AM-Tg mice after 20m-MCAO, and found that the capillary density was significantly correlated with the rota-rod exercise time (Table 1).

In vitro, AM exerted direct anti-apoptotic and neuro-differentiating effects on cultured neuronal cells\(^5\). After 48 hours incubation of normal human neuro-progenitor (NHNp) cells under serum-free apoptotic conditions, in which the number of the cells had decreased to half, the viable cell number was increased in the AM 10\(^{-4}\) mol/liter-treated group. After 7 days incubation of PC12 cells under differentiating condition, both the cell number and the length of neuronal process increased dose dependently as a result of AM treatment. Co-culture with endothelial cells also increased the cell number and the length of the neuronal process. The effect of AM was canceled by AM blockers, PKA inhibitors, and PI3K inhibitors. In this way, AM exerted anti-inflammatory, anti-apoptotic, and vasculo-neuro-regenerative actions in mice model of transient cerebral ischemia (Fig.3). A significant decrease in ssDNA+ apoptotic cells inside and on the border of the ischemic area was observed in AM-Tg mice in association with a reduction in CD45+ inflammatory cells and in situ ROS production during the subacute phase. AM is therefore assumed to reduce delayed neuronal loss through suppression of apoptosis. In addition, we confirmed that AM directly suppresses apoptosis of neuronal progenitor cells in vitro. These findings suggest that AM exerts neuro-protective effects on the ischemic brain by reducing apoptotic neuronal loss through both its direct anti-apoptotic action and indirect effect via anti-inflammation and anti-ROS production. In consistent with the findings, several recent reports have provided evidences for the organ-protective effects of AM against inflammation and oxidative stress\(^\text{1,1,2}\).

In addition, AM promoted vascular regeneration in the mice model of ischemic brain after postoperative day 7, which was consistent with previous reports that showed augmentation of endothelial and vascular regeneration by AM\(^\text{6,7}\). The enhanced vascular regeneration observed after day 7 was followed by subsequent promotion of neurogenesis after day 28. Besides, in vitro studies using cultured neural cells demonstrated the direct effect of AM on neural differentiation via cAMP/PKA- and PI3K/Akt-dependent pathways. Both of the regenerative elements, vascular regeneration and neurogenesis, seemed to contribute for the recovery in motor function after 20m-MCAO (Table 1).

The totality of these findings suggests that the neurogenic action of AM in vivo comprises at least two different mechanisms: a direct action on neural cells through activation of PKA and Akt, and an indirect action caused by enhanced neo-vascularization. In the present study, the number of BrdU/NeuN double positive regenerated neurons was similar between Wt and AM-Tg mice at post-operative day 7, while it was significantly increased in Tg-mice after day 28 (Fig.2D). The time course suggested that AM induced neurogenesis in the ischemic core through the effects which became apparent after postoperative day 7, such as enhanced neo-vascularization; rather than those specifically observed in acute or subacute phase before day 7, including anti-inflammation and anti-apoptosis. Previous reports demonstrated that neurogenesis preferentially occurred in the place surrounded by the vasculature, so-called “vascular niche”\(^\text{1,14}\), where endothelial cells served for neuro-progenitor cells as scaffolds that secret neurogenic factors; and the place was favorable for proliferation and differentiation of neural precursors. Therefore, it is quite reasonable that neurogenesis is closely related with vasculogenesis\(^\text{15}\). For the recovery of motor function after 20m-MCAO, contribution of both of vascular and neural regeneration was indicated. As shown in Table 1, the recovery was well correlated with vasculogenesis rather than neurogenesis, and the finding might be accounted by the fact that the regenerated neurons are very few in the number and their survival rate is very low\(^\text{16}\).

Cerebral ischemia, including stroke, vascular Parkinson’s disease and vascular dementia, is one of the most serious medical problems because it causes critical impairment of activity and quality of daily life. Regenerative medicine is now in the spotlight as a promising therapy to rescue ischemic brain, which has been considered to be irreversible and indicated for no active treatment. The strategy to regenerate ischemic brain is classified in two categories: cell therapy in which neural stem cells are transplanted into brain, and pharmacological therapy that activate endogenous repair systems\(^\text{17,18}\). A variety of cells (e.g. bone marrow cells, embryonic stem cells, and their neural derivatives)
have been used for reconstruction of the neural deficits, including traumatic brain injury, spinal cord injury, Parkinson’s disease and stroke; and beneficial effects have been shown in animal models\textsuperscript{19-21}. A report revealed that intra-arterial transplantation of bone marrow stromal cells in the ischemic rat brain subjected to MCAO resulted in functional recovery associated with survival and neural differentiation of transplanted cells\textsuperscript{22}. However, the therapeutic effect was supposed to be caused by stimulation of endogenous repair systems by transplanted cells; rather than direct replacement and regeneration of ischemic tissue\textsuperscript{23}.

For pharmacological regeneration of the ischemic brain, various humoral factors are anticipated for their therapeutic potential through neurogenic (e.g. basic fibroblast growth factor and epidermal growth factor) and angiogenic (e.g. vascular endothelial growth factor and hepatocyte growth factor) effects\textsuperscript{24-27}. We believe that the vascular hormone AM has several advantages as a therapeutic agent for ischemic brain. We can expect multiple benefits of AM through its neuro-protective and vasculo-neuro-regenerative actions. In addition, AM has already been safely used for human patients with heart failure and pulmonary hypertension without any mention of critical adverse effects resulting from intravenous administration\textsuperscript{28}.

Thus, we are prompted to propose a new strategy to rescue ischemic brain by using vascular hormone AM through its multiple hormonal actions which concurrently lead to anti-inflammatory, neuro-protection and vasculo-neuro-regeneration.

References

13) Palmer TD, Willhoite AR, Gage FH: Vascular niche for adult
110 Mini Review The anti-inflammatory and vasculo-neuro-regenerative roles of adrenomedullin in ischemic brain


