The effect of ADM on human CBF or MCA diameter has not been assessed previously. Data on endogenous production within the brain are conflicting. It seems likely that cerebral endothelial cells produce a high amount of ADM (10, 27, 28). ADM has been shown to pass the blood–brain barrier (BBB) and may be involved in the regulation of BBB function (29, 30). Since ADM receptors are present on both endothelial and vascular smooth muscle cells and possess vasodilatory properties, one would expect ADM to be a strong cerebral vasodilator. However, we found no significant effect on CBF or MCA diameter. This finding contrasts with animal studies, which have all shown vasodilation and increase in CBF. Species differences may partly explain the findings. Thus, the expression of functional ADM receptors might differ between humans and rodents, and the physiological role of ADM is more likely to be regulation of BBB permeability than vasodilation (30–32).

The infusion of ADM resulted in a significant dilation of the superficial branch of the temporal artery compared with placebo. This dilation seemed to be restricted to the cephalic circulation since no effect was seen on the radial (control) artery. The dilation was associated with a concomitant flushing of the face and chest in all but one patient, and a sensation of heat was an often-reported side-effect of the ADM infusion.

Extracranial arterial dilation causes no headache

ADM did not induce a migraine headache in our study population despite its close relation to CGRP and its vasodilatory properties. A possible cause of the failure of ADM to induce migraine headache could be that the dose of ADM was too low. However, plasma ADM_{total} ($ADM_{gly} + ADM_{mature}$) increased 6.5 times and ADM_{mature} 8.8 times. In patients with an altered cerebral circulation after subarachnoid haemorrhage only a four-times increase in ADM plasma concentrations was measured (33). Furthermore, the infusion of a higher dose in the pilot study induced a substantial effect on BP and HR and was deemed unsuitable. We therefore feel confident that the administered dose of ADM was sufficient to induce migraine if the peptide was indeed a mediator of migraine.

Our human model of experimental headache has demonstrated its ability to describe the headache/migraine-inducing potential of several substances, e.g. NO, CGRP and histamine (2, 34, 35). In healthy individuals this headache is monophasic, occurring during and shortly after the infusion. The experimentally induced headache in migraine patients is characterized by a biphasic course with an immediate headache, often similar to the headache experienced by non-migraineurs, and a delayed headache occurring between 1 and 12 h later. The delayed headache fulfilled criteria for MoA in three out of nine patients after CGRP administration (2), in five out of 12 after histamine (36) and in approximately 80% after GTN. In previous studies both intracranial and extracranial dilation were seen, and in spontaneous migraine attacks both the MCA and STA were dilated (37, 38). It remains uncertain which of these territories is most important for migraine induction (39). ADM is the first substance shown to dilate STA without affecting MCA or CBF. Our results indicate that dilation of extracranial vessels is not enough of itself to induce migraine.

In conclusion, the infusion of ADM failed to induce headache or migraine in migraine patients, compared to placebo. It is therefore unlikely that ADM plays a pivotal role in migraine pathogenesis. The vasodilatory properties of ADM were confirmed for the STA and extracranial arterioles (flushing), but neither the MCA diameter nor CBF changed.

Acknowledgements

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Pharmacological stimulation of soluble guanylate cyclase modulates hypoxia-inducible factor-1 α in rat heart

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Pharmacological stimulation of soluble guanylate cyclase modulates hypoxia-inducible factor-1α in rat heart

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Tsuruda T, Hatakeyama K, Masuyama H, Sekita Y, Imamura T, Asada Y, Kitamura K. Pharmacological stimulation of soluble guanylate cyclase modulates hypoxia-inducible factor-1α in rat heart. *Am J Physiol Heart Circ Physiol* 297: H1274–H1280, 2009. First published August 14, 2009; doi:10.1152/ajpheart.00503.2009.—Mechanical load and ischemia induce a series of adaptive physiological responses by activating the expression of O_2-regulated genes, such as hypoxia inducible factor-1α (HIF-1α). The aim of this study was to explore the interaction between HIF-1α and soluble guanylate cyclase (sGC) and its second messenger cGMP in cultured cardiomyocytes exposed to hypoxia and in pressure-overloaded heart. In cultured cardiomyocytes of neonatal rats, either sGC stimulator BAY 41-2272 or cGMP analog 8-bromo-cGMP decreased the hypoxia (1% O_2/5% CO_2)-induced HIF-1α expression, whereas the inhibition of protein kinase G by KT-5823 reversed the effect of BAY 41-2272 on the expression under hypoxic conditions. In pressure-overloaded heart induced by suprarenal aortic constriction (AC) in 7-wk-old male Wistar rats, the administration of BAY 41-2272 (2 $mg \cdot kg^{-1} \cdot day^{-1}$) for 14 days significantly suppressed the protein expression of HIF-1α ($P < 0.05$), vascular endothelial growth factor ($P < 0.01$), and the number of capillary vessels ($P < 0.01$) induced by pressure overload. This study suggests that the pharmacological sGC-cGMP stimulation modulates the HIF-1α expression in response to hypoxia or mechanical load in the heart.

cyclic guanosine monophosphate; hypoxia; mechanical load; angiogenesis; inflammation

THE MYOCARDIUM IS AN elastic network of cardiomyocytes enmeshed in a collagen matrix that connects the myocytes and supporting intramyocardial coronary vasculature. Mechanical load or ischemia induces a series of adaptive physiological responses in the heart [cardiomyocyte hypertrophy, interstitial fibrosis, and angiogenesis (24, 29)]. Hypoxia inducible factor-1 (HIF-1) is one of the most important transcription factors, composed of following subunits: a constitutively expressed HIF-1β and HIF-1α induced by hypoxia, and the latter subunit induces the expression of a number of downstream genes, including that for the vascular endothelial growth factor (VEGF) (26). Expressions of HIF-1α and VEGF are reported to be activated in hypertrophied and failing heart (17, 26, 29, 30), and the increased number of capillary vessels penetrating the interstitial spaces contribute to supply oxygen and nutrients to the cardiocytes for maintaining the structure and function of pressure-overloaded heart (29, 34).

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Guanylate cyclase is an enzyme that converts guanosine triphosphate to cyclic guanosine monophosphate (cGMP). Soluble guanylate cyclase (sGC) activated by nitric oxide has been shown to attenuate cardiovascular remodeling by elevating intracellular cGMP levels (6, 18). We and others have previously reported that the pharmacological stimulation of sGC with BAY 41-2272 attenuated the adverse remodeling associated with systemic or pulmonary hypertension, suggesting that sGC-cGMP activation would be one of the important therapeutic targets for the treatment in the disorders (7, 22, 23). However, the interaction between sGC-cGMP signaling and HIF-1α expression during mechanical load/ischemia in the heart is unknown. Therefore, we sought to examine whether the pharmacological stimulation of sGC-cGMP would affect the HIF-1α-angiogenic pathway in cultured cardiomyocytes exposed to hypoxia and in pressure-overloaded heart.

MATERIALS AND METHODS

The present study was performed in accordance with the Animal Welfare Act and with approval of the University of Miyazaki Institutional Animal Care and Use Committee (2006-014-3, 2002-049-7). It also conformed with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH publication no. 85-23, revised 1996).

Cell culture. Cardiomyocytes were isolated from 1-day-old neonatal Wistar rats as described (38). The cardiomyocytes were cultured on collagen type I-coated culture plates for 48 h with DMEM containing 15 mmol/l HEPES, 10% FBS, 10 μg/ml insulin, 5 μg/ml transferrin, 7 ng/ml sodium selenite, and 0.1 mmol/l bromodeoxyuridine (BrdU) at 37°C in a humidified atmosphere of 95% air-5% CO_2 and further incubated in serum-free DMEM containing the same additives with the exception of BrdU for 48 h. The cells were cultured under normoxic (20% O_2-5% CO_2) or hypoxic (1% O_2-5% CO_2) conditions with or without 5×10^{-5} mol/l BAY 41-2272, a nonhydrolyzable cGMP analog (8-bromo-cGMP, 10^{-3} mol/l; Calbiochem), and a selective protein kinase G inhibitor (KT-5823, 10^{-6} mol/l; Calbiochem) that was added to the culture medium 30 min before hypoxia or BAY 41-2272. Specificity of BAY 41-2272 on concentration-dependent sGC stimulation has been addressed (33), whereas this compound does not stimulate particulate guanylate, adenylate cyclase, or phosphodiesterase 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 [unpublished data, personal communication to Dr. Johannes-Peter Stasch, Bayer Schering Pharma (2, 39)]. The concentrations of 8-bromo-cGMP and KT-5823 were determined by reflecting in accordance with previous studies (14, 35, 41). After the cells had been cultured under normoxic or hypoxic conditions for 8 h, the nuclear extract was extracted according to the manufacturer's recommendations (Pierce). In addition, cultured cardiomyocytes treated with or without BAY 41-2272 for 10 min under normoxia were immediately collected for the cGMP assay as described previously (22).

Animal experiment. Male Wistar rats (7 wk old; Charles River) weighing 200–250 g were housed in a temperature- and light-controlled room (25 ± 1°C; 12:12-h light-dark cycle) for 1 wk before use, with free access to normal rat chow and water. The rats were divided into the following three groups: a sham group (n = 26) and two pressure-overloaded groups with (n = 36) or without (n = 58) BAY 41-2272 treatment. Pressure overload was induced by abdominal aortic constriction (AC) at the suprarenal level as previously described (10, 23). In brief, a 22-gauge needle was placed adjacent to the abdominal aorta proximal to the renal artery and ligated tightly around the aorta and the adjacent needle. The needle was then removed, leaving the vessel constricted to the diameter of the needle. The sham group underwent identical surgical procedures but without constriction of the aorta. The BAY 41-2272 compound, supplied by Bayer HealthCare, was given by gastric gavage at a dose of 2 mg/kg two times a day for 14 days. The dose of BAY 41-2272 was chosen according to our previous study (22, 23), in which 2 mg/kg was the subdepressor dose. The Datascience telemetric system was used to monitor the blood pressure and heart rate of four unrestricted, conscious rats in each study group, as described (23). On *day 14*, the survived rats were anesthetized with pentobarbital sodium and killed by drawing blood from the thoracic aorta. After the whole heart and lung were weighed, left ventricle (LV) was frozen in liquid nitrogen or fixed in 4% paraformaldehyde and embedded in paraffin wax.

Immunohistochemistry and histological analysis. Immunohistochemical staining with HIF-1α, von Willebrand factor (vWF) and monocyte/macrophages (CD68) was performed as reported (22, 36, 37). Tissue sections 3 μm thick fixed in 4% paraformaldehyde were pretreated before incubation with the primary antibodies: HIF-1α, autoclaved at 121°C for 15 min; vWF, covered with proteinase K at 37°C for 15 min; CD68, covered with 0.05% pronase at 37°C for 10 min. Slides were stained with antibodies of HIF-1α (1:6,000, clone H1alpha67; Novus Biologicals), vWF (1:100; DAKOcytomation) or CD68 (1:600, Clone ED1; Chemicon) overnight at 4°C. The slide sections were then incubated with EnVision$^+$ (DAKO) for 30 min, visualized with 0.05% 3,3′-diaminobenzidine containing hydrogen peroxide, and counterstained with hematoxylin. A catalyzed signal amplification system (CSA-DakoCytomation) was used for detecting HIF-1α antigen. Numbers of nuclei stained with HIF-1α, capillary vessels stained with vWF, or monocyte/macrophages stained with CD68 were counted at magnifications of ×200 in a blinded manner.

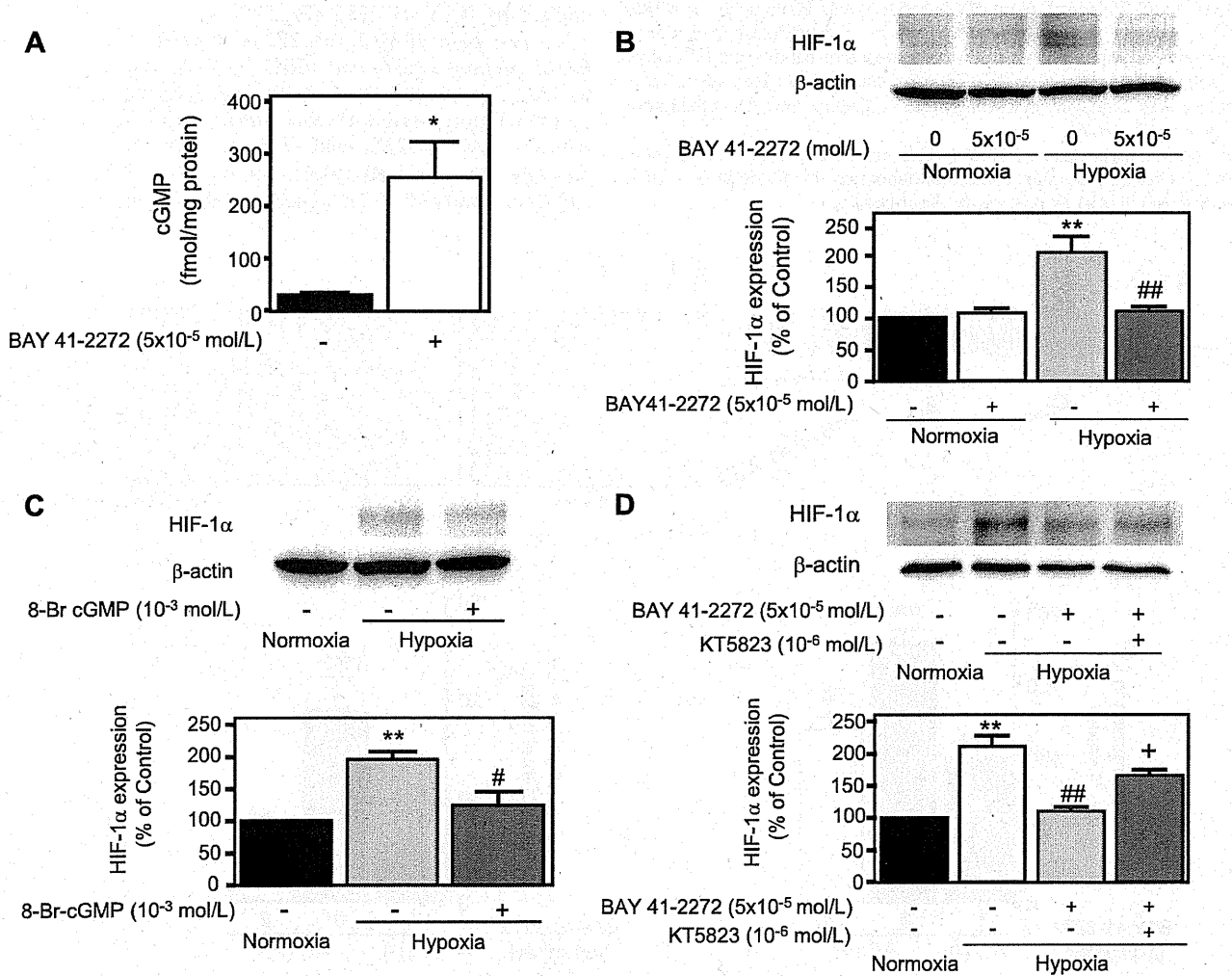

Fig. 1. *A*: Intracellular cGMP content after 10 min of stimulation with BAY 41-2272. *B–D*: Effects of BAY 41-2272 (*B*), the cGMP analog 8-bromo-cGMP (*C*), and the protein kinase G inhibitor KT-5823 in the presence of BAY 41-2272 (*D*) on hypoxia-inducible factor (HIF)-1α protein expression induced by hypoxia in cultured cardiomyocytes. Shown are representative images of Western blots, and values are given as means ± SE for 4-samples. $^*P < 0.05$ and $^{**}P < 0.01$ vs. control/normoxia. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. hypoxia in the absence of any treatment. $^+P < 0.05$ vs. hypoxia in the presence of BAY 41-2272. β-Actin was used for protein loading.

Table 1. *Hemodynamics, heart and lung weight, and cGMP level*

	Sham	AC	AC + BAY 41-2272
Systolic blood pressure, mmHg	122±4	194±5*	183±3*
Diastolic blood pressure, mmHg	89±2	157±8*	155±8*
Heart rate, beats/min	340±42	408±58	378±7
Heart weight/body weight, mg/g	3.2±0.1	4.4±0.2*	4.3±0.2*
Lung weight/body weight, mg/g	4.2±0.1	4.8±0.8	4.0±0.2
cGMP in LV, fmol/mg protein	972±89	1,665±73	2,208±411†

Data are expressed as means ± SE; $n = 4$ for systolic/diastolic blood pressure and heart rate; $n = 6$ (sham), 10 {aortic constriction (AC)}, and 10 (AC + BAY 41-2272) for the other parameters. LV, left ventricle. *$P < 0.01$ and †$P < 0.05$ vs. sham group.

Western blot. Equal amounts of denatured total protein (20 μg) or nuclear extract (10 μg) from the LV or cultured cardiomyocytes were subjected to SDS-polyacrylamide gel as described (36). In brief, the separated proteins electrically transferred onto polyvinylidene difluoride (PVDF) membranes were incubated with 5% skim milk. PVDF membranes were then incubated with a monoclonal antibody against HIF-1α (0.25 μg/ml, clone H1alpha67; Novus Biologicals) or VEGF (0.4 μg/ml, VG1; abcam) followed by a horseradish peroxidase-coupled secondary antibody. Immunoreactive bands were visualized with the ECL Plus detection kit (Amersham), and intensity of each band was analyzed densitometrically (Chemi Doc Documentation System; Bio-Rad).

Radioimmunoassay. cGMP level in the LV and in the cultured cells were determined using a radioimmunoassay kit (YAMASA Cyclic GMP Assay Kit) as previously described (22).

Statistical analysis. All data were analyzed with SPSS software version 11.0 (SPSS). Values are expressed as means ± SE. Differences between two groups were analyzed by Student's *t*-test, and differences between three groups were assessed using one-way ANOVA followed by Scheffé's test. Survival analysis was performed using the Kaplan-Meier method, and statistical significance was accepted at $P < 0.05$.

RESULTS

In vitro effects of BAY 41-2272 and cGMP pathway on hypoxic induction of HIF-1α expression in cultured cardiomyocytes. Figure 1*A* shows that BAY 41-2272 (5×10^{-5} mol/l) significantly ($P < 0.05$) increased the intracellular cGMP level in cultured cardiomyocytes. At this concentration, BAY 41-2272 had little effect on the protein expression of HIF-1α under normoxic conditions, but significantly ($P < 0.01$) inhibited the hypoxia-induced expression of HIF-1α (Fig. 1*B*). A cGMP analog, 8-bromo-cGMP (10^{-3} mol/l), decreased the expression of HIF-1α induced by hypoxia (Fig. 1*C*), whereas the inhibition of protein kinase G by KT-5823 (10^{-6} mol/l) reversed the inhibitory effect elicited by BAY 41-2272 (Fig. 1*D*).

In vivo effects of BAY 41-2272 on systemic blood pressure, heart and lung weight, and cGMP level. As shown in Table 1, the AC significantly ($P < 0.01$) increased the systolic and diastolic blood pressure levels compared with the sham group, whereas BAY 41-2272 had little effect on the elevation of blood pressure induced by pressure overload. In addition, the AC significantly ($P < 0.01$) increased the ratio of heart weight

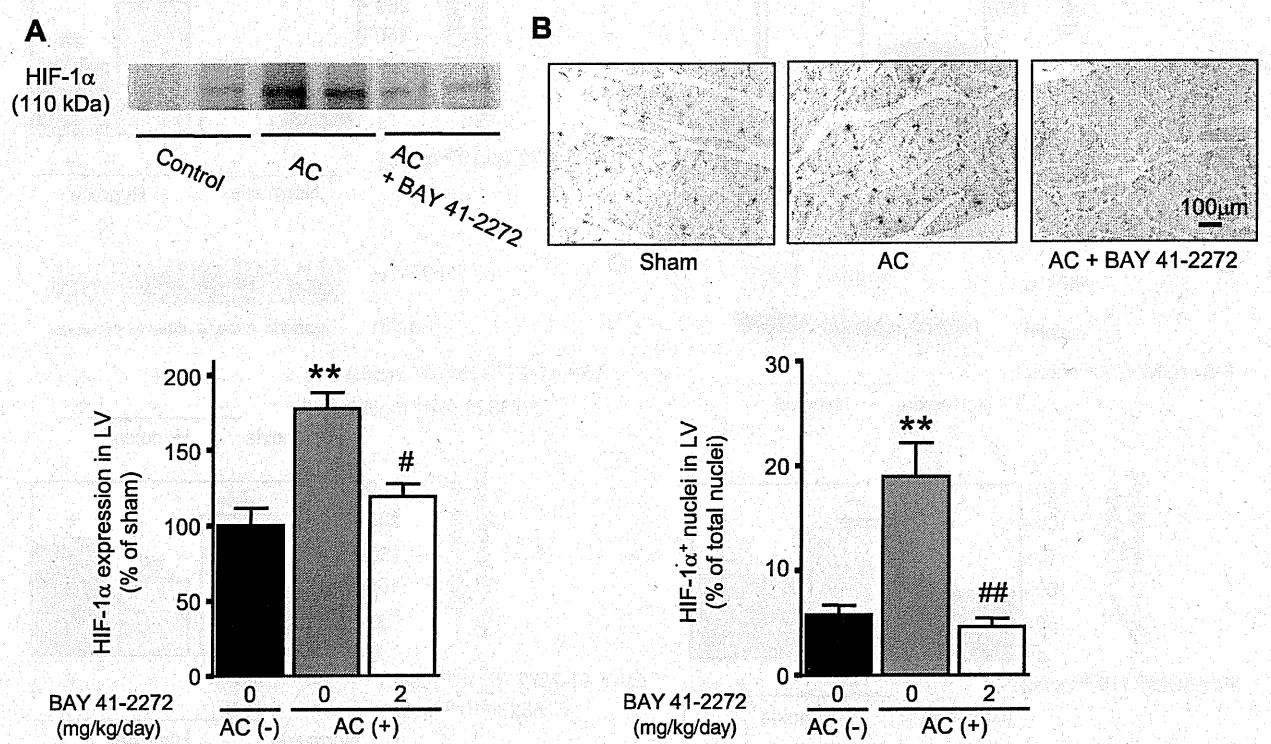

Fig. 2. Effects of BAY 41-2272 on protein expression of HIF-1α (*A*) and number of HIF-1α-positive nuclei (*B*) in the left ventricle (LV). Shown are representative images of Western blot (*A*) and distribution of immunoreactive HIF-1α (*B*) in the sham and aortic constriction (AC) without or with BAY 41-2272 treatment group. Values are shown as means ± SE for 6–10 samples. **$P < 0.01$ vs. the sham group. #$P < 0.05$ and ##$P < 0.01$ vs. the AC group without BAY 41-2272 treatment.

to body weight compared with the sham group. However, it was not affected by the BAY 41-2272 treatment. BAY 41-2272 treatment had a trend to further increase the cGMP level in the LV of AC rats. Neither the heart rate nor the ratio of lung weight to body weight was changed in the respective groups.

Effect of BAY 41-2272 on HIF-1α protein expression induced by pressure overload. Figure 2*A* shows that the protein level of HIF-1α in the LV was significantly ($P < 0.01$) increased by the AC but was significantly ($P < 0.05$) decreased by the BAY 41-2272 treatment. As shown in Fig. 2*B*, the number of nuclei positive for HIF-1α in the cardiocytes was significantly ($P < 0.01$) increased by the AC but was significantly ($P < 0.01$) reduced by the treatment.

Effects of BAY 41-2272 on VEGF protein expression and number of capillary vessels induced by pressure overload. Figure 3*A* shows that the protein level of VEGF in the LV was significantly ($P < 0.01$) increased by the AC but was significantly ($P < 0.01$) decreased by the BAY 41-2272 treatment. Figure 3*B* shows that the AC significantly ($P < 0.01$) increased the number of capillary vessels in the LV; however, BAY 41-2272 significantly ($P < 0.01$) decreased the number by 32%.

Effect of BAY 41-2272 on infiltration of monocyte/macrophages induced by pressure overload. Figure 4 shows that monocyte/macrophages significantly ($P < 0.05$) increased in number, accumulating around the intramyocardial arteries in the LV induced by pressure overload. BAY 41-2272 significantly ($P < 0.05$) decreased the number by 84%.

Survival rate. Figure 5 shows that BAY 41-2272 administration in the AC rats significantly ($P = 0.0395$) reduced the mortality over 14-day periods. Heart failure was the main cause of death, as confirmed by postmortem examination (pulmonary edema or hemorrhage was noted in most of the dead rats).

DISCUSSION

In this study, we report that pharmacological stimulation of sGC-cGMP decreased the hypoxia-induced HIF-1α expression in cultured cardiomyocytes. In addition, the subdepressor dose of BAY 41-2272 modulated the protein expressions of HIF-1α and VEGF and the number of capillary vessels induced by pressure overload.

The beneficial effects of stimulating sGC-cGMP on hemodynamics and remodeling in cardiovascular disorders have been demonstrated by ourselves and others (3, 6, 7, 18, 22, 23). The present study extends our understanding of the important biological action for sGC-cGMP stimulation on improving the survival following the AC. However, the mechanisms by which the stimulation of sGC would affect the remodeling process remain to be defined. In this study, we specifically focused on the interaction between HIF-1α and sGC-cGMP in cardiomyocytes exposed to hypoxia and in LV during mechanical load. As shown, BAY 41-2272 reduced the hypoxia-induced HIF-1α expression in cultured cardiomyocytes, and this was accompanied by an increase in the intracellular cGMP level. In addition, a cGMP analog mimicked the effect of BAY 41-2272 on HIF-1α expression, whereas the inhibition of protein kinase G reversed the effect of BAY 41-2272 on HIF-1α expression induced by hypoxia in these cells. These results suggest that the activation of sGC/cGMP/protein kinase

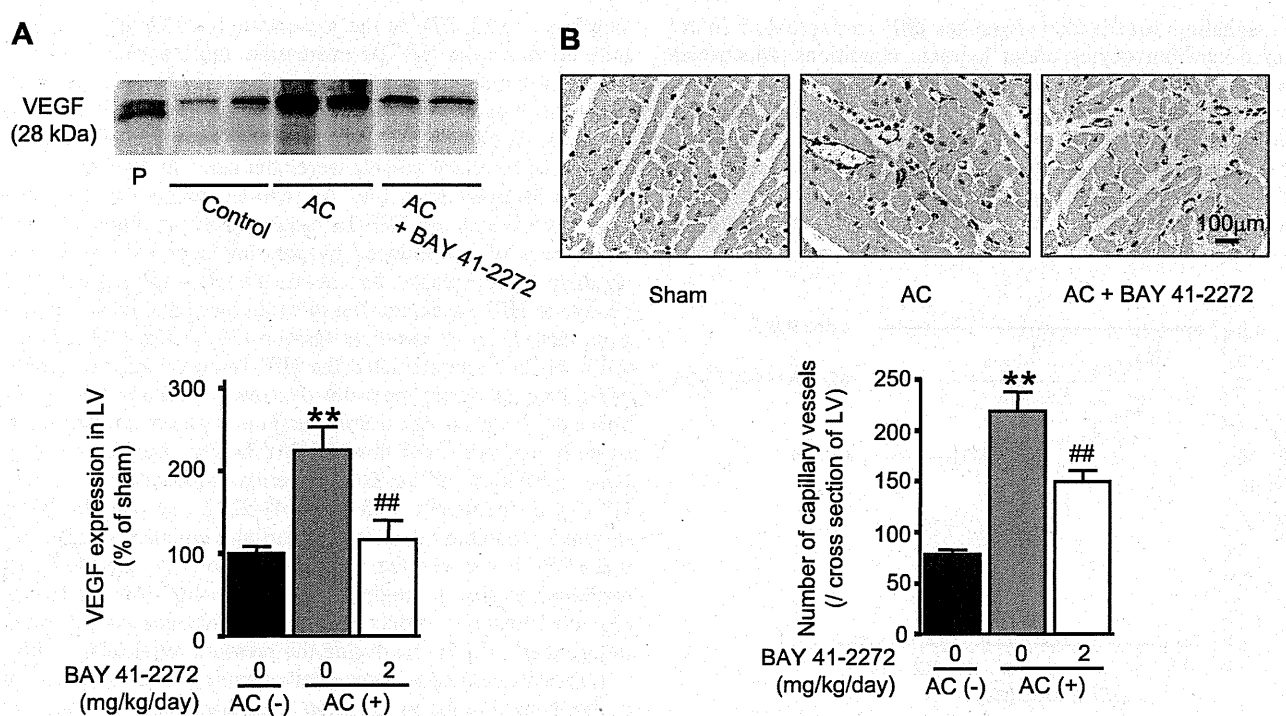

Fig. 3. Effects of BAY 41-2272 on protein expression of vascular endothelial growth factor (VEGF; *A*) and number of microvessels (*B*) in LV. Shown are representative images of Western blot for VEGF (*A*) and distribution of microvessels stained with von Willebrand factor (vWF) (*B*) in the sham and AC without or with BAY 41-2272 treatment group. Values are shown as means ± SE of 6–10 samples examined. $^{**}P < 0.01$ vs. the sham group. $^{\#\#}P < 0.01$ vs. the AC group without BAY 41-2272 treatment. P, positive control (rat kidney) for VEGF.

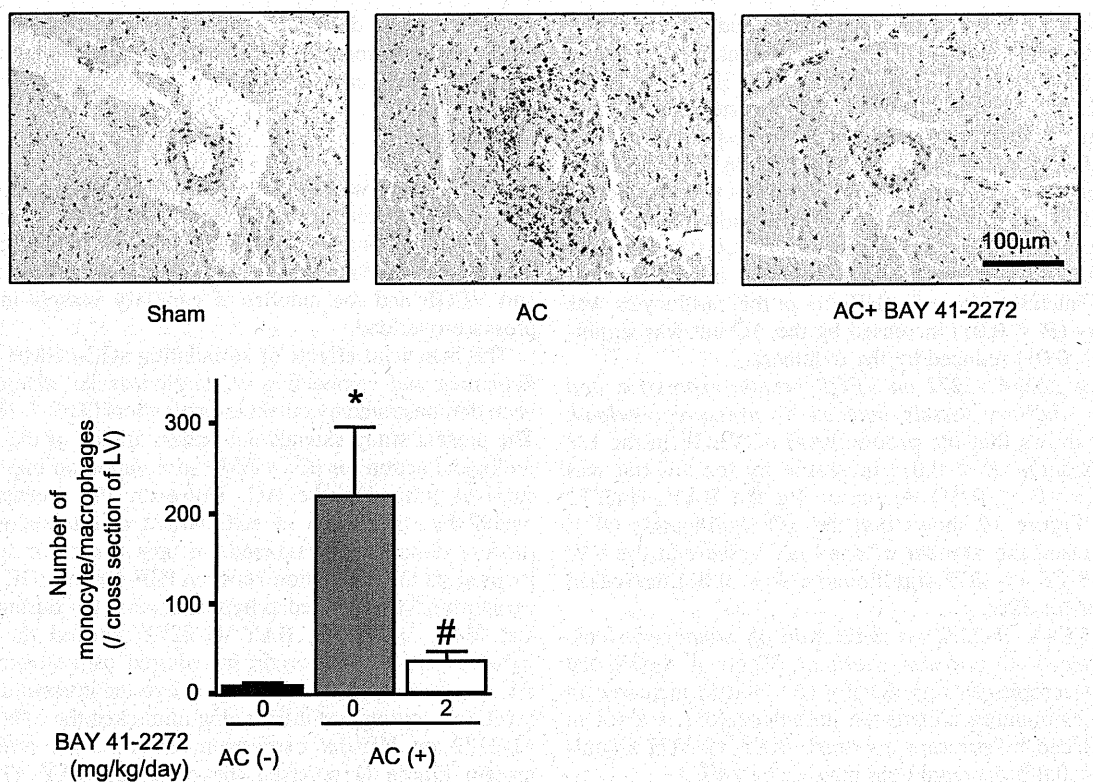

Fig. 4. Effect of BAY 41-2272 on number of monocyte/macrophages in the LV. Shown are representative images of distribution of monocyte/macrophages stained with CD68 antigen in the sham and AC without or with BAY 41-2272 treatment group. Values are shown as means ± SE for 6–10 samples examined. $P < 0.05$ vs. the sham group (*) and vs. the AC group without BAY 41-2272 treatment (#).

G signaling directly downregulates HIF-1α expression in cultured cardiomyocytes under hypoxic conditions. Mechanical load and/or tissue ischemia has been suggested to stimulate the HIF-1α expression during the pressure overload to the heart (17, 29). Our study supports that the HIF-1α expression was increased in the pressure-overloaded LV. On the other hand, the role of sGC-cGMP signaling in modulating HIF-1α and VEGF expressions is reported to be dependent on oxygen supply (1, 5, 20, 27). In the present study, BAY 41-2272 had little effect on the HIF-1α expression under normoxic conditions, but the compound significantly inhibited the expression under the hypoxia in cultured cardiomyocytes. Comparable with this, the protein expressions of HIF-1α and VEGF and the number of capillary vessels were attenuated by the compound in pressure-overloaded LV. As reported previously (30), the immunoreactivity to HIF-1α accumulated in nuclei in the cardiocytes of LV induced by pressure overload, but it was significantly decreased by the treatment, implying that the change in HIF-1α expression in cardiocytes altered the angiogenic activity in a paracrine fashion (29). Thus it seems that BAY 41-2272 counteracted the HIF-1α induction and angiogenic process during pressure overload. Mechanical load induces the multiple signal transductions, stimulating cardiomyocyte hypertrophy and fibrosis (28). Despite the almost complete inhibition of pressure overload-induced increase in HIF-1α by treatment with BAY 41-2272, the corresponding increase in capillary density was partially inhibited, suggesting that HIF-1α was not the only stimulus driving the increased capillary density. It might be explained that other pathways also coordinate to induce the angiogenic gene transcription independent of HIF-1α during the pressure overload (21, 40).

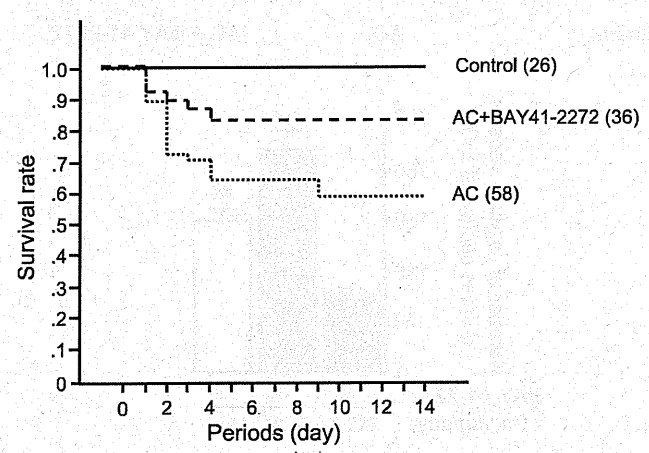

Fig. 5. Effect of BAY41-2272 on survival rate. Kaplan-Meier survival analysis showed a significant reduction of mortality by the BAY41-2272 treatment during the pressure overload (Log rank test; $P = 0.0395$). Parenthesis in the respective group indicates the number of rats.

It appears case-dependent whether angiogenesis is beneficial or detrimental in the progression of cardiovascular diseases (8, 12, 13, 16, 42). As capillary vessels supply oxygen and nutrients to the cardiocytes in response to the demand, angiogenesis would be beneficial for maintaining the structure and

function in ischemic or hypertrophied heart (29, 34). On the other hand, because inflammatory cells are supposed to be recruited from circulating blood (4), one might raise the concern of whether the angiogenic pathway due to the upregulation of HIF-1α and VEGF expression during pressure overload is related to the inflammation and adverse remodeling (8, 9, 31). Zhao et al. (42) have shown that the inhibition of nitric oxide/cGMP increases VEGF and stimulates inflammation and arteriosclerosis surrounding the intramyocardial coronary arteries. Conversely, the present study demonstrates that the stimulation of sGC-cGMP reduced VEGF expression and capillary numbers, concomitant with the decrease in infiltration of monocyte/macrophages in the LV induced by pressure overload. Sano and colleagues (29) reported that HIF-1α-driven angiogenesis is critical for the preservation of cardiac structure and function, preventing the progression of heart failure. Although we found that BAY 41-2272 treatment resulted in the improvement of survival at a relatively early phase by pressure overload, it remains to be elucidated whether the long-term attenuation of the angiogenic pathway by the compound is beneficial in the development of heart failure following cardiac hypertrophy with pressure overload. Further studies are necessary to explore the effect of long-term stimulation of sGC-cGMP inhibiting HIF-1α activity on the remodeling process. We have not assessed the mechanistic insight by which sGC-cGMP inhibited the HIF-1α expression under hypoxia or during mechanical load in this study. However, the nitric oxide signaling pathway has been shown to inhibit the HIF-1 DNA-binding activity and transcriptional activity of HIF-1 target genes in hypoxic cells (11, 20, 32). Therefore, we speculate that the sGC-cGMP stimulation with BAY 41-2272 might have a similar action of nitric oxide under those conditions. In addition, cGMP signaling has been shown to regulate a number of genes with regard to angiogenesis, inflammation, and extracellular matrix (25). Thus it remains unknown whether all of these effects that we observed in this study are explicably mediated by the change in HIF-1α. We have reported that the BAY 41-2272 treatment attenuated the fibrosis induced by pressure overload, accompanied by inhibiting the activity of angiotensin-converting enzyme and the subsequent decrease in the concentration of ANG II in the heart (23). HIF-1α drives not only angiogenesis but regulates the transcription of a number of genes for cell survival/proliferation, matrix metabolism, and vascular tone in a tissue-specific manner (15). Alternatively, the present study might support that the HIF-1α contributes to the syntheses of tissue angiotensin-converting enzyme (19) and extracellular matrix-related genes (9) directly during pressure overload, whereas the sGC-cGMP stimulation would have reversed the remodeling of heart at least in part by modulating the HIF-1α expression.

In summary, this study supports that the HIF-1α expression is upregulated under hypoxia in the cultured cardiomyocytes and in the cardiocytes of pressure-overloaded LV. In addition, our data imply a possible involvement of HIF-1α expression modulated by the pharmacological sGC-cGMP stimulation in regulating the LV remodeling.

ACKNOWLEDGMENTS

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GRANTS

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アドレノメデュリンの発見

Discovery of adrenomedullin

北村和雄

Key words：アドレノメデュリン，PAMP，褐色細胞腫，cAMP，アドレノメデュリン前駆体

1. アドレノメデュリン発見の背景

心房性ナトリウム利尿ペプチドやエンドセリンなどの発見により新しい循環調節機構が明らかになってきたように，複雑で精巧な循環調節機構を解明していくためには，まだ同定されていない新しい循環調節因子を単離し，構造的に明らかにすることが新たな研究の展開のために重要である．宮崎医科大学(現宮崎大学医学部)の松尾・寒川グループからは，50種類近くの新規生理活性ペプチドの発見とそれに続く基礎的・臨床的研究を展開してきており，我が国が世界に誇りうる業績を残している(**図1**)．このような生理活性ペプチドの探索研究の基礎となったのが，1970年代に行われていたオピオイド研究である．脳内に存在する微量の生理活性ペプチドを単離・構造決定するために，それまでの方法論が見直され，改良された．

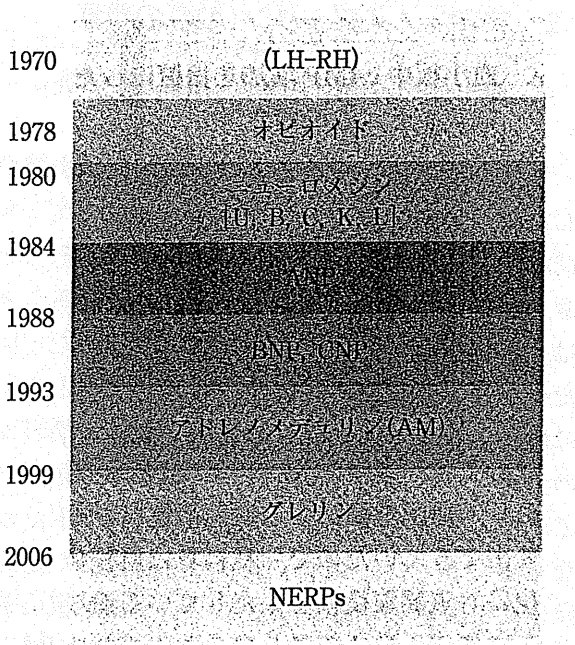

図1　生理活性ペプチドの発見(松尾・寒川グループの貢献)

松尾・寒川グループからは，50種類近くの新規生理活性ペプチドの発見とそれに続く基礎的・臨床的研究を展開してきている．なお，NERPsは松尾・寒川グループ出身の南野らのグループで発見された．

生化学的にモノを扱う研究でまず重要なことは，生体内からモノを抽出する際に，生体内で存在する分子型のままで取り出し，安定化させることである．このために，組織をH_2O中で煮沸後に抽出するという方法が確立された．この方法により内在性のペプチドを生体内で存在する分子型のまま安定化させるとともに，タンパクがプロテアーゼにより分解されて生じるペプチドを最小限に抑えることができ，目的の生理活性ペプチドの精製が容易になった．更に，その当時出てきた高速液体クロマトグラフィや気相式シークエンサーにより，高感度化が進み，比較的少量の組織から生理活性ペプチド探索研究ができるようになった．また，生理活性ペプチドの活性を測定する方法も開発され，摘出平滑筋を用いた非特異的なバイオアッセイにより，

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多くのニューロメジン類が発見された．このように生理活性ペプチド探索研究の方法論が確立されるときに，私は大学院生として在籍しており，今考えると，得難い経験と勉強ができたと感じている．そして，大学院4年のときに，私の直接指導をしてくれていた寒川先生が心房性ナトリウム利尿ペプチドを数カ月で単離・構造決定し，グローバルに活躍されるようになるのを見て，私も将来は自分自身の生理活性ペプチドを発見して，研究を展開したいと思うようになった．

2. アドレノメデュリン(AM)の発見

a. 血小板中cAMP増加を指標にした新しいアッセイ法の確立[1]

大学院終了後しばらく大学病院の医員を経験した後，米国に留学し，1988年に帰国して，再び第1内科で仕事をするようになった．そのとき，私自身が教室や大学のために役立てることは，松尾先生・寒川先生より指導していただいたペプチド探索研究の方法論を生かすこと以外にはないと考えていた．当時は高血圧・循環器の分野では，エンドセリンやBNPが発見されており，血圧を調節する生理活性ペプチドはかなり出てきていたので，私自身は血栓症や動脈硬化にも重要な役割を果たしている血小板に着目して研究を進めることにした．当時の宮崎医科大学の内科学第一講座では，アンジオテンシン変換酵素阻害薬の血小板機能に及ぼす影響についての研究がされていたこともあり，血小板凝集機能の検査の機器がそろっていたことも，血小板に作用するペプチドの探索を考えた理由の一つである．

最初のうちはウサギやヒトの血小板に対する凝集能で生理活性ペプチドの探索を試みたが，再現性や感度の面でうまくいかなかった．それで，血小板中cAMP上昇が血小板機能を抑制することをヒントにして，ラット血小板の浮遊液(50μL)にペプチド検体(50μL)を加え37°Cで反応させた後，産生されたcAMPをラジオイムノアッセイ(RIA)で測定し，その増加を活性の指標とする方法を作成した．本アッセイ法は簡便で，高感度ではあったが，未知の生理活性ペプチドの探索法とするためには，再現性に問題があった．

再現性の悪い理由の一つとして，アッセイごとに調製する血小板のばらつきが考えられ，最初にそれについて検討したが改良はみられなかった．当初著者らは，反応時間を長くするとcAMP産生量も増加するので，アッセイの精度も上がるものと考え，5分あるいは10分間の反応時間で行っていた．しかし，5分と10分での結果を比較すると，反応時間の短い5分の方で，血小板中cAMP濃度がコントロールと比較して高く，更にばらつきが少ない傾向が認められたため，次に反応時間について細かく検討を行った．その結果，30秒と極端に短時間の反応を行うことにより，高感度で再現性の高い結果が得られることが明らかになった．血小板中では検体刺激によりcAMPはごく短時間で上昇するが，その後反応性にphosphodiesteraseが活性化し，血小板中cAMPが減少してしまうためだと考えている．

このようにして我々は，簡便かつ高感度で再現性の高いアッセイ法という，未知物質検索のための新たな方法論を手にすることができた．研究を開始して，新たなアッセイ系を確立するために，既に2年近くの歳月を要していた．

b. 血小板中cAMP増加活性を指標にしたペプチドの系統的検索[2]

苦労して確立した上記のアッセイ法を用いて，未知のペプチドの検索を開始した．しかし，新しいアッセイ法が確立できたからといって，簡単に新規ペプチドが発見できたわけではなかった．我々はそれまでの微量生理活性ペプチドの検索には，大量の組織の入手が容易であるブタの組織を出発材料として用いており，新しいアッセイ法による検索においてもブタの脳，消化管，心臓などの抽出物を用いて検討していたが，それらの組織からは新規ペプチドへの手がかりは得られていなかった．

当時の宮崎医科大学第1内科では，褐色細胞腫の患者は1年に1症例あるかないかであった．全くの偶然だと思われるが，血小板中cAMP増

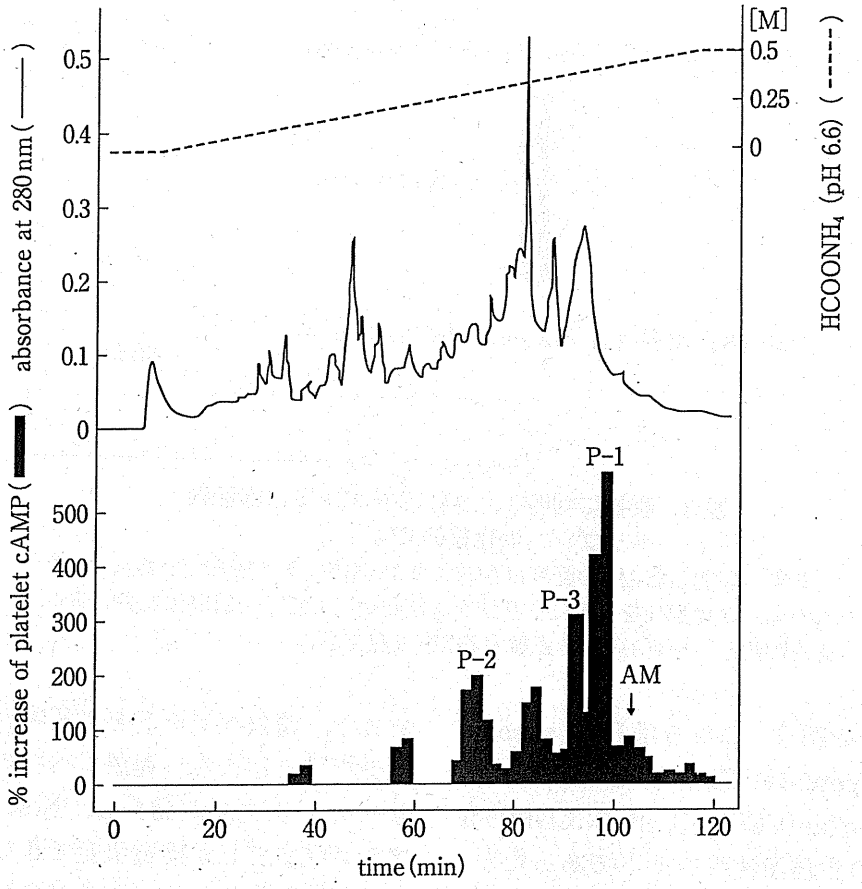

図2 アドレノメデュリンの発見

ヒト褐色細胞腫抽出液の強塩基性ペプチド分画の陽イオン交換(CM)HPLC. 血小板中cAMP増加活性を有したペプチドとして, VIP, CGRP-I, CGRP-IIが最初単離され, アドレノメデュリン(AM)は矢印の小さなピークより発見された.

加を指標とした生理活性ペプチドのアッセイ系が確立できたころに, 4人の褐色細胞腫の患者が続けて入院し, それらの患者さんの協力もあり, 大量の褐色細胞腫の組織を入手することができた. 褐色細胞腫患者からの摘出腫瘍組織の抽出物についても検討したところ, 褐色細胞腫組織では正常副腎髄質(剖検組織)の約5-10倍という顕著なcAMP増加活性を有することが明らかとなった.

褐色細胞腫からのペプチド抽出については, 普通の組織はH_2Oで煮沸するのに対し, 1M酢酸中という酸性条件下で煮沸している. これは, 褐色細胞腫に含まれる大量のカテコールアミンとペプチドが副反応(恐らく酸化的にトリプトファンに反応する)を起こすことを防ぐためである. このことは, 大学院生時代に松尾・寒川研究室でウシ副腎髄質や褐色細胞腫からBAMペプチドやアドレノルフィンが発見されるのを見ていて知っていたことでできたことであり, ここでも大学院時代の経験が生かせた.

血小板中cAMP増加活性を強く示すPC組織(140g)を出発材料として, cAMP増加活性ペプチドの系統的検索に取りかかった. 粗ペプチド抽出物をSP-Sephadex(陽イオン交換樹脂)で分離すると, 活性の約90%は強塩基性画分に存在することから, 本画分をSephadex G-50によるゲル濾過で分離し, 分子量約3,000-5,000の領域に溶出された活性画分を更に陽イオン交換HPLCで分離した. その結果, **図2**に示すように4つの主要な血小板中cAMP増加活性と多くの小さな活性が認められた. 主要活性ピークのP-1, P-2, P-3について逆相HPLCを繰り返して精製を進め, それぞれ純品として単離した後, シークエンサーによりアミノ酸配列の決

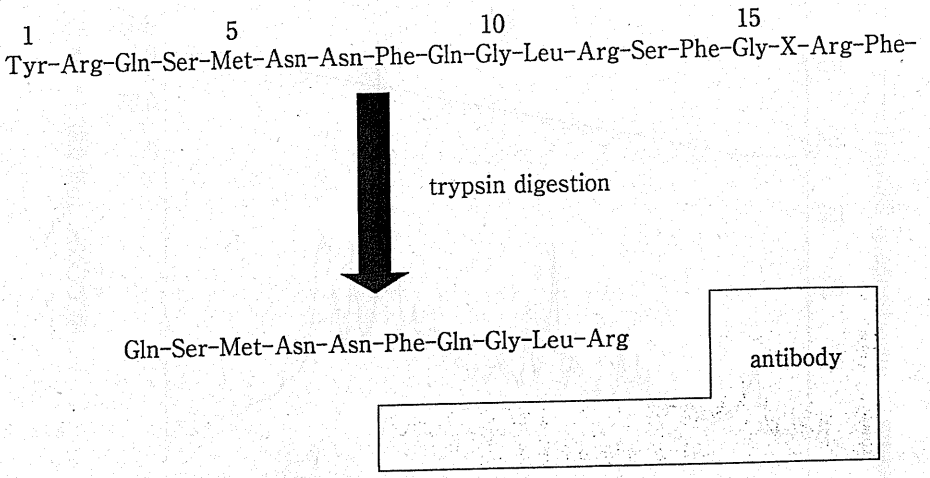

図3 最初に判明したAMのN末アミノ酸配列とトリプシン消化後のRIA

最初に判明したAMの配列はN末より18個のアミノ酸までであった．そのため，この配列をもとにトリプシン消化後にAM[3-12]が生成されることを利用し，AM[3-12]を認識するRIAを確立した．

定を行った．その結果，これらはP-1: vasoactive intestinal peptide(VIP)，P-2: calcitonin gene related peptide(CGRP-I)，P-3: CGRP-IIとすべて既知の生理活性ペプチドであった[1]．新たなアッセイを用いたことで，新規ペプチドが発見できることを期待していただけに，既知ペプチドであったことにがっかりしたが，これらのペプチドはいずれも強力な血管拡張性の降圧ペプチドとして知られており，本アッセイを用いることにより新しい降圧ペプチドの発見が期待された．引き続き，マイナー活性ピークについても順次単離，構造決定を進めたが，それらについてもVIPやCGRPのMet残基の酸化など既知ペプチドの修飾の結果生じた関連ペプチドであった．そのため，寒川先生からのアドバイスもあり，VIP，CGRPなどのRIAを確立し，VIP，CGRPの免疫活性を示さずに，血小板中cAMP増加活性を示す分画の単離・構造解析を行い，数十種類にも及ぶ既知ペプチドやその関連物質の単離，構造決定を虚仮(こけ)の一心で続けた．そして，強塩基性部に溶出される小さな活性ピークからついに新たな生理活性ペプチドを見いだした(図2)．

c. アドレノメデュリンの構造決定[2]

しかし，やっとの思いで精製できたペプチドは約140gの褐色細胞腫よりわずか20pmolであった．精製ペプチドを気相式シークエンサーで分析したところ，運悪くペプチドシークエンサーのトラブルが発生し，N末より18番目のアミノ酸までしか構造決定できなかった(図3)．この段階で，新しいペプチドの存在が判明したが，18番目までのアミノ酸配列からは既知の生理活性ペプチドとの相同性が全く認められなかった．この時点で心配したこととしては，精製したペプチド自体が血小板中cAMP増加活性を有している場合は問題ないが，精製したペプチドピークに含まれる別の血小板中cAMP増加活性を有した極少量のペプチドが強力な活性を示している可能性を完全には否定できなかった．そこで，判明したアミノ酸配列より，図3で示すようなトリプシン消化後に生ずる10個のアミノ酸からなるペプチドに対するRIAを確立した．本RIAと血小板中cAMP増加活性を指標に，AMを多量に含有していた別の褐色細胞腫40gより再び精製を行い，300pmolのAMを精製することができた．当時は，300pmolでは52残基のペプチドの全構造決定を行うのに決して十分すぎる量ではなかったが，松尾・寒川グループで確立されていた微量ペプチド構造解析法により，アミノ酸分析，還元カルボキシメチル化後のN末端からのアミノ酸配列分析，酵素消化により断片化されたペプチドの配列分析，C末端

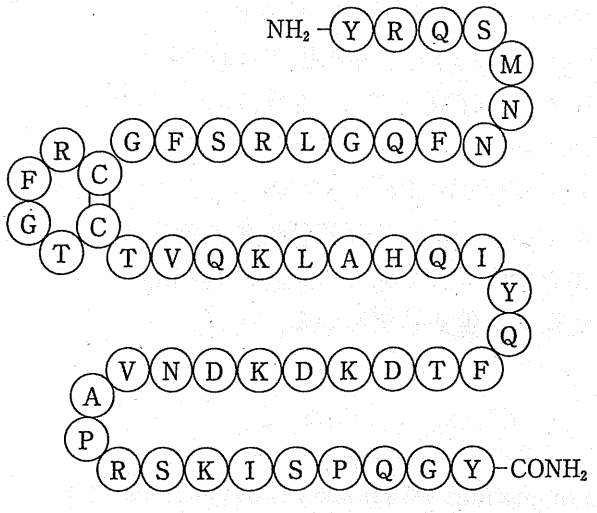

図4 ヒトアドレノメデュリン(AM)の全構造

ヒトAMは52個のアミノ酸よりなるペプチドで6個のアミノ酸よりなるリング構造をもち，C末端はアミド化されている．

部合成ペプチドとの比較によるC末端アミド構造の決定などを行った．このようにして，ついに新規ペプチドの(S-S)結合を含む全構造を決定することができた(図4)．

本ペプチドは副腎髄質由来の褐色細胞腫より発見され，また正常副腎髄質(adrenal medulla)にも高濃度存在することより，アドレノメデュリン(adrenomedullin: AM)と命名された．AMは52個のアミノ酸よりなり，1個のジスルフィド結合をもつ新しい生理活性ペプチドであることが判明した．C末端は他の幾つかの生理活性ペプチドでみられるようにアミド化されていた．

d. AM前駆体の構造とPAMPの発見[3-5]

AMの生合成機構や生体内での役割を明らかにするため，AM遺伝子のcDNAクローニングを行い，前駆体の構造を明らかにした．**図5**に示すように，ヒトAM前駆体は，21個のシグナルペプチドを含む185個のアミノ酸よりなり[3]，AMのシークエンスの両サイドは典型的なプロセッシングシグナル(LysArgもしくはArgArg)で囲まれていて，C末のTyrに続くGlyはアミドの供与体になると考えられる．また，AM配列のN末端側には生理活性ペプチドの構成性(constitutive)分泌の典型的なプロセッシングシグナルとされているArg-X-Arg-X-X-Argが存在している．このことは後に明らかにされてきたように，AMが副腎髄質などの内分泌組織以外の心血管系をはじめ多くの組織で発現されていることを考えると興味深い事実である．

更に，AMの前駆体には新しい生理活性ペプチドと考えられる興味あるアミノ酸配列が認められた[3]．シグナルペプチドに続くproadrenomedullinのN末の20個のペプチドは，C末端のArgに続き，Gly-Lys-Argという典型的なアミド化シグナルが認められた．この事実は，N末の20個のペプチドのC末端がArgアミド構造を有した新しい生理活性ペプチドとして生合成される可能性を示唆し，このペプチドをproadrenomedullin N-terminal 20 peptide(PAMP)と命名した．我々は，PAMPをブタ副腎髄質や褐色細胞腫より単離構造決定すること

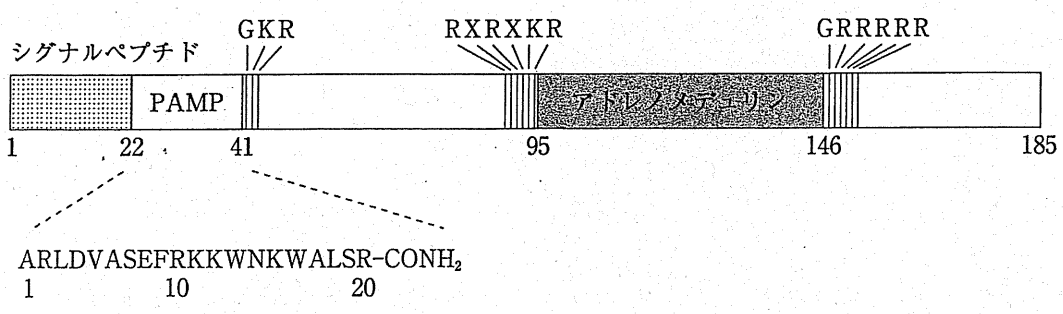

図5 ヒトアドレノメデュリン前駆体の模式図

ヒトAM前駆体は，21個のシグナルペプチドを含む185個のアミノ酸よりなり，AMのシークエンスの両サイドは典型的なプロセッシングシグナル(LysArgもしくはArgArg)で囲まれていて，C末のTyrに続くGlyはアミドの供与体になると考えられる．また，AM以外に，ヒトAM前駆体(proadrenomedullin)のN末の20個のペプチドは，C末端がArgアミド構造を有したペプチドとして生合成され，proadrenomedullin N-terminal 20 peptide(PAMP)と命名した．

で，生体内での存在を明らかにした[4,5]．また，PAMPも降圧活性を有したペプチドであることが判明したが，降圧の機序はAMとは異なることが明らかになり，両ペプチドが協調して生体内調節に関与している可能性が示唆されている．

おわりに

AMは副腎髄質由来の褐色細胞腫より発見されたが，その後の研究により，AMは心血管系で産生される重要な循環調節因子であり，更にAMによる新たな循環調節機構が明らかになってきた．現在までに，2,000以上のAMに関する論文が報告され，単なる循環調節因子ではなく，生体に必須な因子であることも明らかになっている．更に，循環器疾患治療薬としての応用も期待されており，臨床での実用化を目指した研究も進められている．

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話題のホルモン・受容体・酵素：最近の知見から

アドレノメデュリン(AM), PAMPとその受容体

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Introduction

アドレノメデュリン(adrenomedullin；AM)は1993年に褐色細胞腫組織より発見された強力な血管拡張性の降圧作用を有する生理活性ペプチドであり，降圧系の重要な循環調節因子の1つであることが明らかになってきた。AMは副腎髄質以外にも心血管系組織を含め，生体内の幅広い組織で生合成されている。AMの作用としては，降圧作用以外にも，レニン・アルドステロン分泌抑制，ナトリウム利尿，心筋細胞肥大抑制，抗酸化，臓器保護作用などの多彩な作用を有している。AMの受容体に関しては，7回膜貫通型受容体であるcalcitonin receptor like receptor(CRLR)とreceptor activity modifying protein 2(RAMP2)およびRAMP3が共発現することでAM受容体を形成する。

一方，AM前駆体からはproadrenomedullin N-terminal 20 peptide(PAMP)が別の生理活性ペプチドとして生合成される。AMとPAMPともに降圧作用を示すが，両ペプチドの作用機序は異なっており，共通の前駆体から生合成される2つのペプチドが協調して循環調節などに関与していると考えられる。

AMは血中にも存在しており，心不全，高血圧・腎不全，敗血症性ショックなどの患者では血中AM濃度が疾患の重症度に従って上昇していることが明らかになっている。これらの疾患においては，AMは降圧系の循環調節因子として作用するばかりではなく，抗炎症・臓器保護因子としての役割も果たしていることが示唆されている。さらに急性心筋梗塞などの疾患モデル動物では合成AM投与により，病態が改善することが示されており，AMが新しい医薬品として開発できる可能性が示唆されている。

アドレノメデュリン(AM)とPAMPの構造，分布，遺伝子

AMはヒト褐色細胞腫組織から発見された強力な血管拡張性ペプチドであり，特徴として分子内に6個のアミノ酸よりなるリング構造とC末端のアミド構造を有している(図1)[1,2]。ヒトAMは52個のアミノ酸よりなり，calcitonin gene related peptide(CGRP)やアミリンと

一部相同性を有し，1つのファミリーを構成している(図2)。

最近，遺伝子側から検索することで，AMに続くアドレノメデュリン2(AM2)/intermedin(ITM)の存在が明らかにされた[3,4]。遺伝子構造から推定されるヒトAM2/ITMの構造は，47個のアミノ酸からなり，AMと同様に6個のアミノ酸よりなるリング構造があり，C末はアミド化されている(図2)。AMとの構造上の相同性はそれほど高くなく，約25%である。今後，AM2の生体内での役割を明らかにすることで，AM研究の新たな展開が期待される。

AMはヒト副腎由来の褐色細胞腫から発見されたが，心房，心室，肺，腎臓などの組織にも副腎に匹敵するmRNAを認める(図3)。特に，血管内皮・平滑筋細胞での遺伝子発現は副腎を凌駕する。このほか，脳内や多くの末梢組織にも存在しており，AM mRNAの体内分布は実に広範である。

ヒトAMの前駆体の構造は，図4に示すように21個のシグナルペプチドを含む185個のアミノ酸よりなる[5]。AM配列の両サイドは典型的なプロセッシングシグナル(LysArgもしくはArgArg)で囲まれていて，C末のTyrに続くGlyはC末端アミド構造の供与体になると考えられる。

AMの前駆体には新しい生理活性ペ

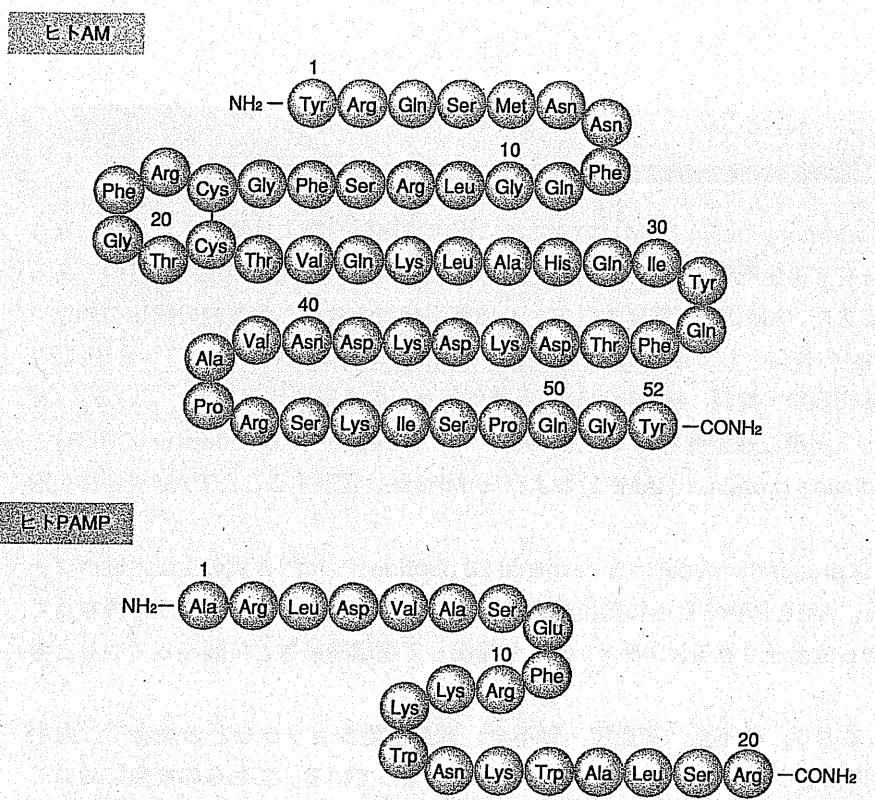

図1 ヒトアドレノメデュリン(AM)とproadrenomedullin N-terminal 20 peptide(PAMP)のアミノ酸配列(文献2より引用)

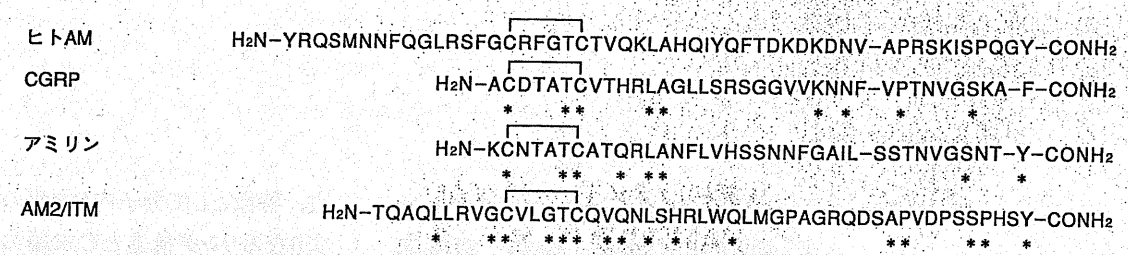

図2 ヒトアドレノメデュリン(AM)，CGRP，アミリン，アドレノメデュリン(AM)2/intermedin(ITM)のアミノ酸配列の比較(文献5より改変引用)

*：ヒトアドレノメデュリンと共通のアミノ酸を示す。
⌐¬：6個のアミノ酸よりなるリング構造。

プチドと考えられる興味あるアミノ酸配列が認められた[5]。シグナルペプチドに続くproadrenomedullinのN末の20個のアミノ酸からなるペプチドは，C末端がArgアミド構造を有した新しい生理活性ペプチドがproadrenomedullinよりAMとは別に生合成されることが明らかとなり，このペプチドはPAMPと命名された(図1，4)。

ヒトAM遺伝子は11番染色体短腕上に存在し，プロモーター領域には転写調節因子の結合部位が数多く存在する[5](図4)。転写開始点近傍のactivator protein(AP)-2の結合部位の集団(-68，-33bp)や核内因子-インターロイキン6(NF-IL6)の結合部位(-93，-85bp)の存在は，AP-2とNF-IL6が血管内皮のAM遺伝子を制御する重要な転写因子であることをうかがわせる。特に，NF-IL6は炎症の誘導物質(リポ多糖や腫瘍壊死因子，IL-1など)によって活性化され，これらの多くが血管内皮・平滑筋細胞でのAM産生を強力に促進する。

AMの作用

AMには多彩な作用が明らかにされたが，その特徴的な作用は強力な降圧作用である。AMを麻酔下ラットに単回静注すると，30～60分間持続する強力な血管拡張を伴った降圧が観察される[1]。AMの心拍出量増加作用はAMが心臓に直接作用している可能性も考えられている。さらに，培養心筋細胞を用いた研究から，AMはアンジオテンシンIIによる心筋蛋白合成能の活性化を著明に抑制し，アンジオテンシンIIによる心筋肥大を抑制する作用があることが示唆されている[6]。

AMは腎動脈に投与したとき，強力な水・ナトリウム利尿作用を示す。また，AMは副腎皮質からのアルドステロン分泌抑制作用，ラット下垂体前葉からの

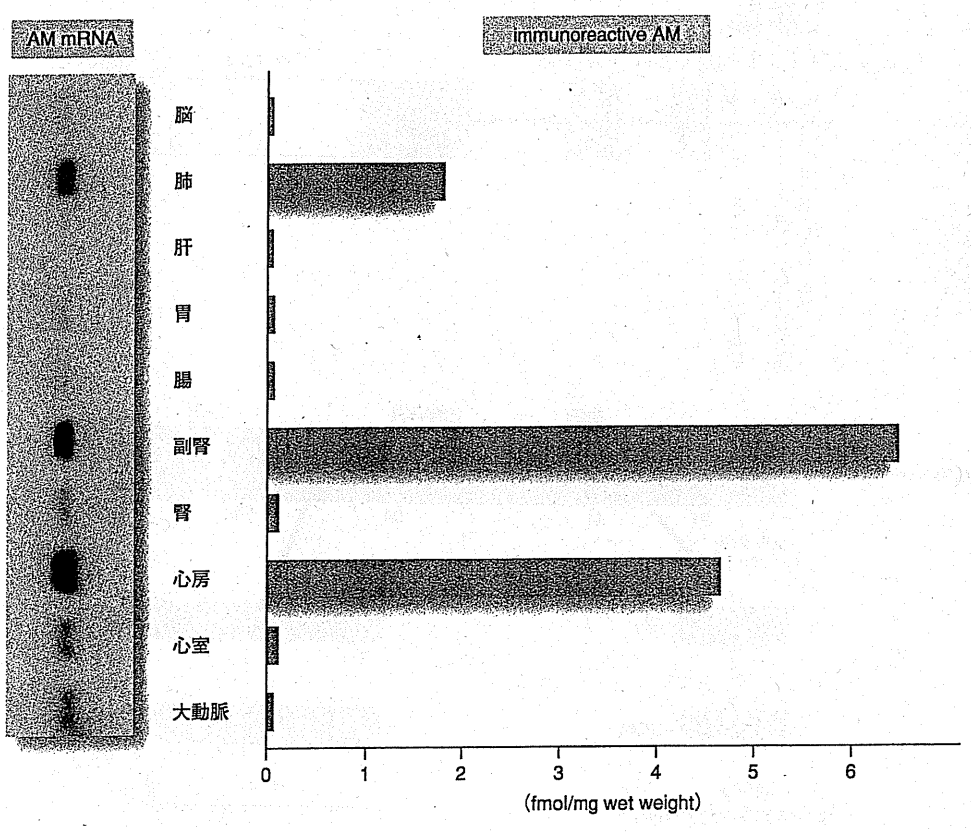

図3 ラット各組織のAMの濃度(右)と遺伝子発現(左)
(文献5より引用)

副腎皮質刺激ホルモン(adrenocorticotropic hormone；ACTH)分泌抑制作用を有す。さらに，覚醒ラットの脳室にAMを投与すると，飲水行動の抑制，塩分摂取行動の抑制，バソプレシンの分泌抑制などが報告されている。このように，AMは末梢性にも中枢性にも水・電解質バランスの調節に関与している可能性がある。

現在までに明らかにされているAMの作用を昇圧因子であるアンジオテンシンⅡと比較してみると，AMのほとんどの作用はアンジオテンシンⅡに拮抗することがわかる(表1)[6]。AMがアンジオテンシンⅡに拮抗する内因性因子として，心臓・腎臓疾患における病態に重要な役割を果たしていることが予想される。

AM受容体

AMとCGRPは，産生部位や作用部位は異なるが，生理作用は類似しており，またAM作用の一部はCGRPアンタゴニストであるCGRP(8-37)でブロックされることから，AM受容体には独自の受容体とともにCGRPと共通の受容体が存在するものと推定されていた。その後，AM受容体に関しては，

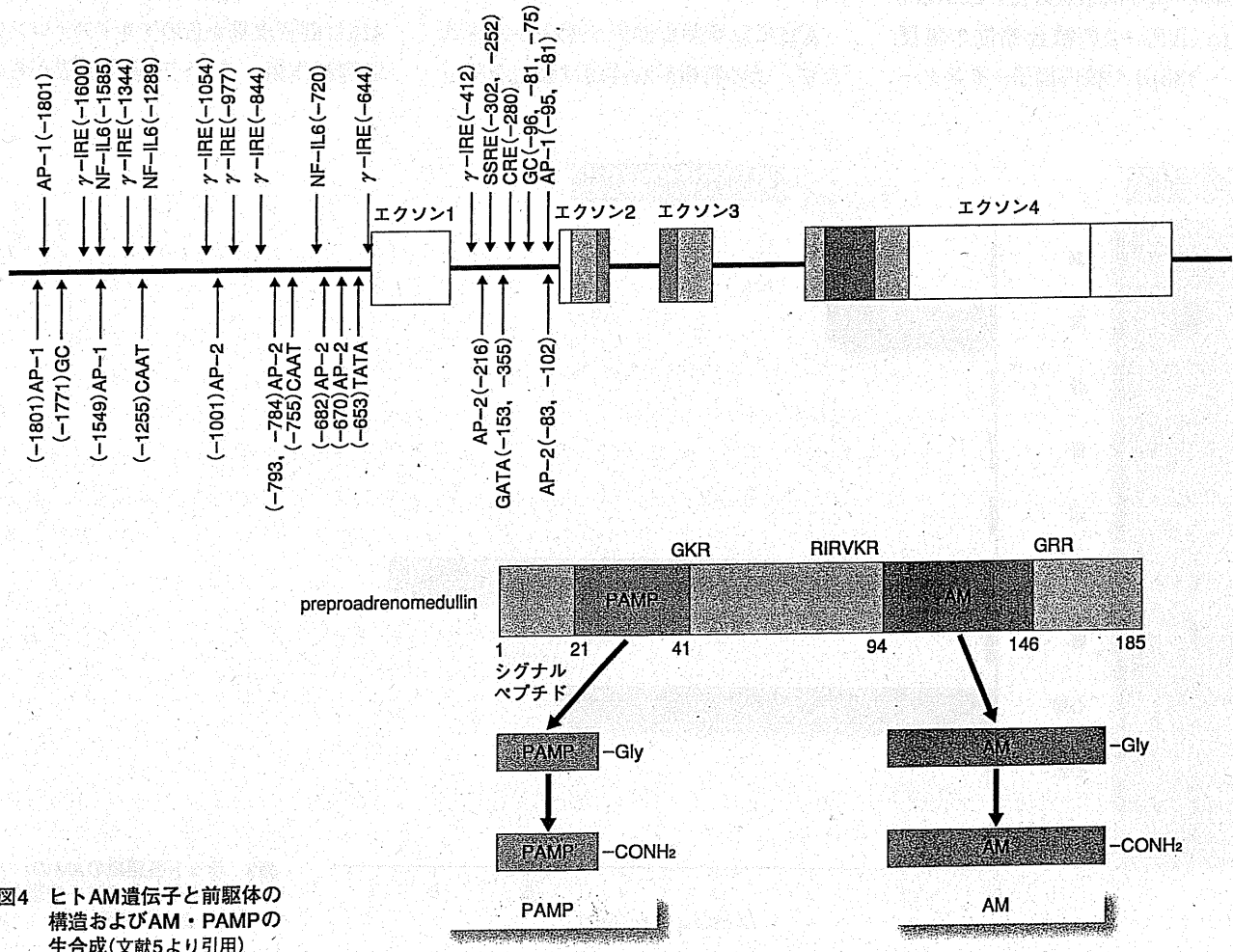

図4 ヒトAM遺伝子と前駆体の構造およびAM・PAMPの生合成(文献5より引用)