Leucine Aminopeptidase M–Induced Reductions in Blood Pressure in Spontaneously Hypertensive Rats

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Leucine aminopeptidase M significantly reduced blood pressure for up to 40 minutes when infused intracerebroventricularly into anesthetized spontaneously hypertensive rats (SHR) from a mean±SEM of 190±4 to 94±7 mm Hg and also in normotensive Wistar-Kyoto (WKY) rats from 138±5 to 68±8 mm Hg. Cerebrospinal fluid levels of angiotensin II (Ang II) and III were measured by radioimmunoassay and indicated drops with leucine aminopeptidase M infusion in SHR (from 36±6 to 11±1 pg/100 μl) and in WKY rats (from 33±9 to 13±1 pg/100 μl). Pretreatment with the specific angiotensin receptor antagonist [Sar1, Thr8]Ang II (sarthran) significantly diminished the subsequent leucine aminopeptidase M–induced decreases in blood pressure in SHR and facilitated recovery to base level blood pressure and heart rate in both strains. Thus, exogenous application of leucine aminopeptidase M into the brain lateral ventricles of SHR is temporarily effective at reducing blood pressure, and this effect appears dependent on the brain angiotensinergic system. This treatment also reduced blood pressure in WKY rats; however, pretreatment with sarthran was reasonably ineffective at preventing subsequent leucine aminopeptidase M–induced decreases in blood pressure. (Hypertension 1989;13:910–915)

Spontaneously hypertensive rats (SHR) and the stroke-prone SHR (SHRSP) appear to possess a dysfunctional brain renin-angiotensin system that contributes to their hypertension. Schelling and colleagues measured elevated levels of renin in brain areas associated with catecholaminergic nuclei in SHR, and Ganten et al observed greater turnover of brain angiotensin than in normotensive rats. Our laboratory has measured deficiencies in brain aminopeptidase activity in SHR, which may explain their heightened sensitivity to intracerebroventricularly injected angiotensin II (Ang II) and angiotensin III (Ang III). Electrophysiological studies also indicate a significantly increased sensitivity to microiontophoretically injected Ang II and Ang III in the paraventricular nucleus of SHR as compared with Wistar-Kyoto (WKY) normotensive rats.

In the present investigation, we hypothesized that the exogenous application of aminopeptidases in SHR may temporarily correct this brain deficiency in peptidase activity and reduce blood pressure. This approach has recently been used intrarterially in SHR with limited success by Mizutani and colleagues. These investigators administered bolus injection of an aminopeptidase A (P-APA)–rich preparation purified from human placenta. In a preliminary investigation, we compared the efficacy of P-APA and a commercially available leucine aminopeptidase microsomal (LAP-M), prepared from hog kidney microsomes in two ways. First, each of these aminopeptidase preparations were incubated with [125I]Ang II or [125I]Ang III for up to 60 minutes, and degradation of the ligand was measured by high-performance liquid chromatography (HPLC) analysis (see Materials and Methods). Second, each preparation was infused intracerebroventricularly at several doses, and blood pressure was measured. Both procedures indicated that the commercial aminopeptidase was more effective, and therefore it was used in the present investigation. Given the hypothesized deficiency in brain aminopeptidase activity in SHR, we predicted that intracerebroventricular infusion of aminopeptidase M would significantly lower blood pressure in SHR and, to a lesser degree, in the WKY normotensive rat.
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FIGURE 1. Percent radiolabel [125I]angiotensin II (A II) or [125I]angiotensin III (A III) remaining intact during a 60-minute incubation period at 37° C with aminopeptidase prepared from human placenta (P-APA) (top panel) or leucine aminopeptidase prepared from hog kidney microsomes (LAP-M) (bottom panel). The ability of LAP-M to degrade [125I][Sar1,Thr3]angiotensin II was also examined.

Materials and Methods
Aminopeptidase Activity
The P-APA-rich preparation (prepared by Dr. S. Mizutani’s laboratory) and LAP-M (Sigma Chemical Co., St. Louis, Missouri; L5006), prepared from hog kidney microsomes (LAP-M) (bottom panel). The ability of LAP-M to degrade [125I][Sar1,Thr3]angiotensin II was also examined.

Animals and Instrumentation
Mature male SHR and WKY rats (derived from stock purchased from Taconic Farms, Inc., Germantown, New York) 120-180 days of age were bred and maintained in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited vivarium on a 12:12 hour photoperiod initiated at 7:00 AM and a temperature of 22±1° C. Food and water were available ad libitum except during testing, which occurred between 12:00 noon and 5:00 PM. Each rat was initially injected with diazepam (2.5 mg/kg, Parke-Davis, Morris Plains, New Jersey) followed 10 minutes later with ketamine (100 mg/kg, Bristol-Myers Co., New York, New York) and prepared with a left femoral artery catheter (PE-50, Clay Adams, Parsippany, New Jersey) and an intracerebroventricular guide cannula stereotaxically positioned just above the roof of the right lateral ventricle as previously described.9 Blood pressure and heart rate measurements were monitored via the femoral artery catheter connected to Gould transducers (model P23Id, Gould Statham, Oxnard, California) and a Grass Instruments polygraph (model 7b, Grass Instr. Co., Quincy, Massachusetts) equipped with a Grass tachygraph (model 7P4h).

Protocol 1: Intracerebroventricular Leucine Aminopeptidase M Infusion
Eight rats from each strain were used for blood pressure testing. After the establishment of a 10-minute stable, anesthetized base level blood pressure, a 24-gauge stainless steel hypodermic tubing injector was inserted into the guide cannula such that it extended 1-2 mm beyond the tip of the guide, thus penetrating the roof of the lateral ventricle. The injector was connected to a 1-ml syringe by a 60-cm length of PE-20 tubing that was graduated in l-/xl units (i.e., 1.1 cm/μl) to facilitate the monitoring of the infusion rate of 2 μl/min (infuser, model 355, Sage Instruments, Cambridge, Massachusetts). Each rat received a control infusion of artificial cerebral spinal fluid (aCSF) at a rate of 2 μl/min for 10 minutes and LAP-M (5 munits/2 μl aCSF) again at 2 μl/min for 10 minutes. The order of the infusions were counterbalanced such that four rats from each strain received aCSF first and the other four rats initially received LAP-M. Sufficient time was allowed between infusions to regain base level blood pressure.

Protocol 2: Cerebral Spinal Fluid Sampling for Angiotensin Levels
An additional 16 rats from each strain were prepared as described above and were similarly tested; however, eight rats from each strain received only LAP-M (5 munits/2 μl aCSF at a rate of 2 μl/min for 10 minutes). The other eight rats were infused with aCSF (2 μl/min for 10 minutes). At the point of maximum blood pressure reduction to LAP-M, minutes 15–20 after initiation of infusion as determined in protocol 1, a 200–300 μl CSF sample was obtained via cisterna magna tap as previously described.10 The tap needle penetrated the cisterna 2–3 mm, and the CSF was collected by gravity feed into an ice-cold, siliconized (Sigmacote, SL-2, Sigma Chemical Co.) culture tube containing 100 μl acetonitrile (ACN) to stop peptide degradation. Collection required approximately 5–10 minutes. The volume of CSF was then measured, and additional
acetonitrile was added until it reached equivalent volume with the sample. The samples were then dried for 12 hours (dryer, model SVC-100, Savant Instr., Inc., Farmingdale, New York) and stored at 
−20°C until radioimmunoassay (RIA) was performed to determine angiotensin levels. The RIA has been described in detail. The antiserum was equivalently reactive to Ang II and Ang III as determined by self-displacement and as a result reflected the total levels of biologically active angiotensin in the samples.

Protocol 3: Intracerebroventricular Pretreatment With Sarthran Followed by Leucine Aminopeptidase M Infusion

A final 16 rats from each strain were surgically prepared as described above and received an intracerebroventricular pretreatment of the specific angiotensin receptor antagonist sarthran. One subgroup of eight rats from each strain received a dose of 10 mmol/min, and the other eight rats received a dose of 20 mmol/min in a volume of 2 μl aCSF/min for 15 minutes. At 5 minutes into sarthran infusion, a 10-minute intra-arterial infusion of phenylephrine hydrochloride (P6126, Sigma Chemical Co.) was initiated to offset the hypotensive effect of sarthran in both strains. The dose of phenylephrine was 0.02 mg/kg/min in 0.15 M NaCl infused at a volume of 50 μl/min via a three-way connector (TC-20/3, Small Parts, Inc., Miami, Florida) located 20 cm from the point of entry into the femoral artery. This procedure allowed for the uninterrupted recording of blood pressure during phenylephrine infusion. Preliminary testing indicated that the effects of intra-arterial infusion of phenylephrine dissipated within 10 minutes of the termination of infusion. The effects of intracerebroventricularly infused sarthran have been shown to persist for at least 30 minutes, and once association occurs between the angiotensin molecule and its receptor, the peptide appears inaccessible to peptidase activity. After completion of these pretreatment infusions, rats were allowed a 5-minute recovery period, and then LAP-M was intracerebroventricularly infused at a dose of 5 munits/2 μl aCSF/min for 10 minutes. Blood pressure was monitored for an additional 80 minutes, or until it returned to base level. Next, an intracerebroventricular infusion of aCSF (2 μl/min for 15 minutes) was substituted for sarthran, and after a 5-minute latency, an intra-arterial infusion of sterile 0.15 M NaCl (50 μl/min for 10 minutes) was initiated. At the conclusion of combined intracerebroventricular and intra-arterial infusion, a 5-minute delay was instituted followed by intracerebroventricular LAP-M infusion as described above. Once again blood pressure was monitored for 80 minutes. The order of the pretreatments was counterbalanced such that four rats within each subgroup of each strain initially received sarthran combined with phenylephrine, and the other four received the control infusions first.

Statistical Analyses

In protocol 1, changes in blood pressure were calculated by subtracting the 10-minute mean arterial base level for each group before infusion from the maximum change induced by the infusion of aCSF or LAP-M. These data were analyzed by a 2 (groups)×2 (treatments) analysis of variance (ANOVA) with repeated measures on the second factor. The data of protocol 2 concerned with CSF angiotensin levels were analyzed by a 2 (strain)×2 (treatments) ANOVA. The datasets for blood pressure and heart rate from protocol 3 were analyzed by a 2 (strain)×3 (pretreatment) ANOVA with repeated measures on the second factor. Significant effects were further evaluated by Newman-Keuls post hoc tests at a significance level of 0.01.

Results

Leucine Aminopeptidase M Infusion and Cerebral Spinal Fluid Angiotensin Levels

Figure 2 presents the effects of intracerebroventricular infusion of aCSF or LAP-M in SHR and WKY rats. There were significant differences among the groups (F=61.90, df=3,28, p<0.001) because of LAP-M-induced changes in blood pressure in both SHR and WKY rats. There were strain differences in the maximum decreases in mean arterial blood pressure produced by LAP-M infusion (F=204.37, df=1,28, p<0.0001). Post hoc analyses indicated that SHR showed a greater absolute drop in blood pressure than did the WKY rats (mean±SEM, 95.8±7.2 and 69.9±7.7 mm Hg, respectively). The interaction of groups and treatments was also significant (F=82.17, df=3,28, p<0.001), which indicates a differential effect of treatments with aCSF or

![Figure 2. Arterial blood pressure changes (mean±SEM) from base level during and after a 10-minute intracerebroventricular infusion of artificial cerebrospinal fluid (aCSF) (2 μl/min) or leucine aminopeptidase M (LAP-M) (5 munits/min) in Wistar-Kyoto (WKY) rat and spontaneously hypertensive rat (SHR) groups. Base level mean blood pressures before infusion were 140.7±4.7, 189.4±4.3, 138.1±5.3, and 190.0±4.4 mm Hg for WKY rat and SHR groups administered aCSF and the WKY rat and SHR groups treated with LAP-M, respectively.](http://hyper.ahajournals.org/doi/abs/10.1161/01.hyp.13.6.912?journalCode=hyp)
LAP-M on blood pressure in these strains. The RIA results indicated significant drops in CSF angiotensin concentrations in both strains comparing the intracerebroventricular infusion of LAP-M with aCSF ($F=74.45$, df=1,28, $p<0.001$). Specifically, in the WKY rats levels dropped from 33±9 pg/100 µl after aCSF infusion to 13±1 pg/100 µl after LAP-M infusion. Respective values for SHR were 36±6 to 11±1 pg/100 µl CSF. There were no differences between strains or interaction of strain and treatment. The aCSF control infusion RIA values were somewhat higher than are usually reported. We suspect that this was due to a combination of the multiple surgical preparations, lack of the use of a muscle relaxant, the duration required by the protocol before CSF sampling, and perhaps the increase in ventricular volume due to the intracerebroventricular infusions. Since CSF samples were always denatured with 50% acetonitrile and processed through small pore C18 columns that restricted protein elution, no LAP-M either in active or inactive forms should have been in the final RIA samples. Even if some protein did make its way into the final samples, the effects would be insignificant in light of the large excess of bovine serum albumin (BSA) typically used in the RIA.

Sarthran Pretreatment Followed by Leucine Aminopeptidase M Infusion

Figure 3 presents the blood pressure results of a 15-minute intracerebroventricular pretreatment with aCSF, 10 or 20 nmol/min sarthran, followed by intracerebroventricular LAP-M infusion in SHR and WKY rats. There was a strain difference ($F=11.88$, df=1,14, $p<0.005$) in that SHR revealed a greater overall drop in blood pressure across treatments than did WKY rats. There was also a significant effect of treatments ($F=17.00$, df=2,28, $p<0.0001$); pretreatment with aCSF resulted in the greatest subsequent LAP-M-induced drop in blood pressure across strains, whereas pretreatment with the two doses of sarthran resulted in equivalent inhibition of the LAP-M-induced drop in blood pressure. Finally, there was a strain–treatment interaction effect ($F=11.06$, df=2,28, $p<0.005$). Post hoc analyses indicated that pretreatment with aCSF had a minimal effect on subsequent LAP-M-induced decreases in blood pressure in SHR and WKY rats, whereas pretreatment with either 10 or 20 nmol/min sarthran greatly inhibited the subsequent LAP-M-induced drop in blood pressure in SHR but had very little influence on WKY rats.

Figure 4 presents changes in heart rate as a result of intracerebroventricular pretreatment with aCSF, 10 or 20 nmol/min sarthran, followed by intracerebroventricular LAP-M infusion in SHR and WKY rats. There was a strain difference ($F=11.59$, df=1,14, $p<0.005$) with SHR showing the greatest drop in heart rate across pretreatments, the pattern of LAP-M-induced changes in heart rate were very similar in members of the two strains over the conditions we used.

Discussion

The suggestion that angiotensinas play a role in the pathogenesis of hypertension was originally offered by Hickler et al and Itskovitz et al.
SHR

aCSF>LAP-M
SAR-10>LAP-M
SAR-20>LAP-M

1000
Infusion
Post-Infusion
FIGURE 4. Heart rate changes (mean±SEM) from base level during and after a 10-minute intracerebroventricular infusion of leucine aminopeptidase M (LAP-M) in separate groups of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Mean heart rates after pretreatment infusions for the SHR groups with artificial cerebrospinal fluid (aCSF), SAR-10, or SAR-20 were 373±13, 327±19, and 342±18 beats/min, respectively. The values for the WKY rat groups were 315±22, 311±15, and 311±21 beats/min, respectively. Base level mean heart rates during the 5-minute delay period before LAP-M infusion for the SHR groups pretreated with aCSF, SAR-10, or SAR-20 were 375±10, 333±12, and 346±15 beats/min. Comparable values for the WKY rat groups were 312±22, 320±8, and 316±19 beats/min.

However, data to support this hypothesis as it pertains to a central mechanism are only now becoming available. Along these lines our laboratory has been investigating procedures to manipulate the half-lives of brain-synthesized Ang II and Ang III initially by employing aminopeptidase inhibitors. In vitro studies have previously shown that amastatin specifically inhibits APA, which is responsible for cleaving aspartate and converting Ang II to Ang III,\textsuperscript{15,16} whereas bestatin inhibits aminopeptidase B,\textsuperscript{17} which cleaves arginine, thus converting Ang III to its hexapeptide. It should be noted that both amastatin and bestatin also inhibit leucine aminopeptidase, which hydrolyzes a variety of amino terminal residues and aminopeptidase M.\textsuperscript{16,18} Intracerebroventricularly delivered amastatin and bestatin produce pressor responses with SHR that are more responsive than those of normotensive rat strains.\textsuperscript{19,20} Furthermore, intracerebroventricular pre-treatment with bestatin extends the half-lives of exogenously injected [\textsuperscript{125}I]Ang II and [\textsuperscript{125}I]Ang III in the ventricular space,\textsuperscript{21,22} which suggests that these aminopeptidase inhibitors are exerting their influence via extending the half-lives of endogenously synthesized angiotensins.

From these initial efforts came our present investigation in which aminopeptidases were infused into the brain ventricles in an attempt to reduce blood pressure in SHR. In both SHR and WKY rats, the results indicate a substantial reduction in anesthetized blood pressure that lasted up to 40 minutes after termination of infusion. This LAP-M-induced reduction in blood pressure in SHR appears angiotensinergic dependent because pretreatment with the specific angiotensin receptor antagonist sarthran reduced the hypotensive effect of subsequent LAP-M infusion. However, sarthran’s effect was minimal in WKY normotensive rats. Concomitant heart rate results indicated a greater drop in SHR treated with LAP-M as compared with the WKY rats; however, no differential effect due to sarthran pretreatment was measured. It should be noted that the mean base level heart rates of groups of SHR were greater than those of WKY rat groups (i.e., 351 and 316 beats/min, respectively).

The only previous group to employ exogenous applications of aminopeptidases was Mizutani and colleagues.\textsuperscript{8,23} The intra-arterial bolus injection of a placenta-derived aminopeptidase A–rich preparation reduced blood pressure in anesthetized SHR by up to 50 mm Hg. The duration of the effect was not measured. These investigators attributed this drop in blood pressure to degradation of endogenously synthesized Ang II. Scott et al\textsuperscript{24} previously observed that the blood pressure of pregnant SHR and WKY rats declines on about day 18 of gestation. Mizutani et al\textsuperscript{8} reported progressive elevations in plasma APA activity throughout normal human pregnancy with the highest levels at term, and they concluded that the plasma hypotensive factor noted by Scott et al in the pregnant SHR was probably of APA origin.

The results of the present investigation are generally supportive of our hypothesis that SHR suffer a deficiency in brain aminopeptidase activity because the intracerebroventricular infusion of LAP-M did reduce blood pressure to a greater degree in SHR than in WKY rats. However, substantial drops in blood pressure were noted in members of both strains (SHR, 190–94 mm Hg; WKY, 138–68 mm Hg). The unusually high initial levels of CSF angiotensin immunoreactivity in the groups of WKY rats and SHR (330 and 360 pg/ml, respectively) are presently without explanation beyond the possibilities previously noted. Reported CSF levels range from 28 to 32 pg/ml.\textsuperscript{10,25} The significant drops in CSF Ang II and Ang III levels after LAP-M infusion, accompanied by reductions in blood pressure and heart rate, are consistent with the notion that the endogenously synthesized angiotensins are being degraded by the exogenously infused LAP-M. How-
ever, the observations that the initial levels of CSF Ang II and Ang III were approximately equivalent in the groups of WKY rats and SHR, and similar drops in these levels that occurred with LAP-M infusion, would not be expected if only SHR suffered from a deficiency in brain aminopeptidase activity. Clearly, further investigations are necessary to determine an optimal dose of this enzyme and to isolate the active components of this commercial preparation.

In summary, our present results suggest that when intracerebroventricularly infused, a leucine aminopeptidase can be effectively used as a hypotensive agent in anesthetized, SHR and normotensive WKY rats. Pretreatment with a specific angiotensin receptor antagonist diminished the hypotensive effect of LAP-M in SHR, thus supporting the notion that this effect is dependent on the brain angiotensinergic system. However, similar decreases in CSF angiotensin levels were measured in LAP-M-treated SHR and WKY rats as compared with an artificial CSF–treated control condition, thus questioning the specificity of this treatment. These results do encourage the further evaluation of the role of aminopeptidases in the central control of blood pressure.

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