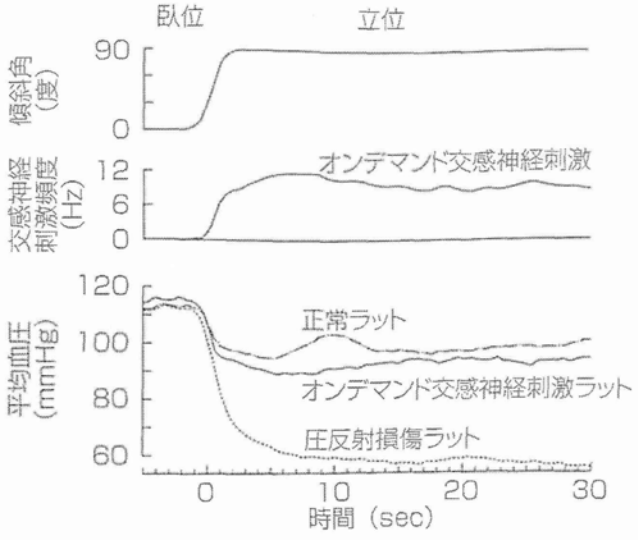

図1．血圧安定化治療のラット（腹腔神経節刺激）でのコンセプト証明

ある．

これらの低血圧は脳幹部にある血管運動中枢が果たす血圧安定化機能（圧反射系の機能）の低下により起こる．その治療には圧反射系の機能再建が必要であり，機能再建はオンデマンド治療（低血圧が起こる条件下のみで作動して行う治療）の実現を意味する．低血圧を治療するために昇圧薬を投与することは臥位時の高血圧を誘発するので適切な治療法ではない．

本治療のコンセプトは「オンデマンド神経刺激により機能しない生体の血圧安定化機能を再建」することである．次項に述べる基礎研究の結果，このコンセプト実現には生体自体の制御原理である，常時測定した血圧の目標値からのずれを検出して交感神経を刺激するフィードバック制御を再現することで可能であるという見通しが得られた．

2）基礎研究

生体のような複雑な制御系が制御工学で多用する線形の伝達関数で表現できるのかは議論の多いところであった．しかし著者らの研究によりこれが可能であり，非線形の静特性と線形の動特性（伝達関数）の組み合わせを用いれば生体の人為的な制御にも応用できることが知られてきた．

そこで動物において圧反射系の静特性と動特性を詳細に調べ，これをもとに血圧安定化の手法を設計した．予め生体の圧センサ部分を他の動脈系から分離し（開ループ），圧受容器にかける圧（頸動脈圧）を自由に変化できるようにした．静特性は頸動脈圧を階段状に増減させ，動特性は頸動脈圧を乱数的に変化させることによって求めた．圧反射系全体のゲインと動特性は圧反射系がフィードバック動作をする際の挙動に影響を与え，外乱（起立など）による血圧低下からの回復の速度，安定性（振動などの有無），程度を決めることが明らかになった．

3）動物でのコンセプト証明

血圧安定化のために必要な交感神経刺激の条件を決める手法は以下のように設計した．刺激条件は実時間で求めた．まず正常ラットを用い前項で述べた方法で，頸動脈洞（両側）に乱数的な圧変化を加えた際の血圧変化を記録し，圧反射系全体の動特性を求めた．次に交感神経に電極を装着して，交感神経を乱数的に変化する刺激頻度で電気刺激した際の血圧変化を記録し，交感神経刺激頻度に対する血圧応答の動特性を求めた．これらの伝達関数の比を用いることによって，血圧センサによって記録した血圧（とその履歴）に応じてどのように交感神経の刺激頻度を設定すればいいかを算出した．

圧反射系を無効化した麻酔下のモデルラット（圧反射損傷ラット）を用い，血圧をカテ先血圧計で連続記録して，上記の算法により必要な刺激頻度で交感神経を電気刺激した．起立時の血圧維持が正常ラットと同程度に可能かどうかを動物実験によって検討した[1,2]．交感神経の刺激部位は静脈収縮による効果を期待して腹腔神経節を直接に電気刺激した．

図1はラット起立時の血圧変化を示す．圧反射損傷ラットで起立時に起こる重症低血圧は交感神経のオンデマンド刺激による血圧安定化治療で抑制できた．

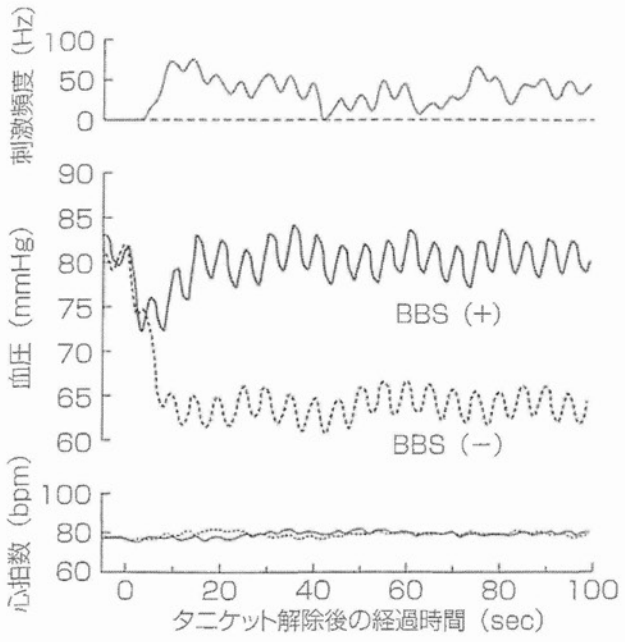

図2. 血圧安定化治療の術中低血圧患者（硬膜外腔刺激）でのコンセプト証明.（文献5より許可を得て引用）

臨床応用を考慮した場合，腹腔神経節への電極装着や固定は極めて困難である．そのために同様の効果が得られる他の刺激方法を開発する必要がある．幸いにも上記の刺激頻度の算出は（刺激条件自体はちがっても），刺激部位や直接，間接の刺激の区別にかかわらず利用できる．臨床応用に向け，脊髄硬膜外腔での間接的な交感神経刺激について検討した．ネコを用いた脊髄硬膜外腔のオンデマンド電気刺激実験[3]で同様に血圧の安定化が可能であった．

4）ヒトでのコンセプト証明

動物でのコンセプト証明ができたため，倫理委員会の承認のもと，ヒトでのコンセプト証明を行う臨床研究を行った．

佐藤ら[4,5]は膝関節置換術中の止血用タニケットの開放時に不可避である重症の低血圧を抑制する試みを行った．一回の手術で通常複数回のタニケットの開放が行われるので，オンデマンド血圧安定化を行うか行わないかを無作為に決め，両者での血圧の低下度とその時間経過を比較した．血圧安定化は脊髄硬膜外腔での電気刺激により行った．

図2はこの比較結果を示す．血圧安定化を行わない場合（中段，BBS（−））は高度の血圧低下が持続した．一方，血圧安定化を行うことで（中段，BBS（+））血圧低下は一過性，軽度に留まった．血圧低下の時間は20秒以内であった．

一部の患者では薬剤を用いた昇圧速度の検討を行ったが，昇圧に要する時間は約10倍であり，神経性制御の即時性がオンデマンド治療に果たす役割の重要性が示された．

また砂川らの研究室[6]では経皮的な電気刺激により間接的に血圧の上昇が可能であることを示した．この刺激法を用い，脊髄損傷患者の起立性低血圧を防止する血圧安定化治療を開発した．幅広い刺激強度が可変となるよう刺激条件の最適化を行い，刺激強度は電流量により変化させた．図3に血圧安定化治療の例を示す．脊髄損傷患者では上体を少し起こすだけでも高度の低血圧が起こるが（上段，制御開始以前），血圧安定化治療の制御を開始するとほぼ20秒以内に血圧が元のレベルに復した．（上段，制御開始以後）．

3. 迷走神経刺激治療

1）治療コンセプト

慢性心不全は生体制御が不適応となる代表的な疾患である．このことは近年の心不全薬物治療のパラダイムシフトの基礎となっており，心不全に伴う交感神経系やレニン・アンジオテンシン系の過剰活動を抑制する薬剤（β交感神経遮断薬，アンジオテンシン変換酵素阻害薬，アンジオテンシン受容体遮断薬）が生存率を含む予後改善をもたらしていることに現れている．

慢性心不全での制御不適応は，交感神経の活動過剰に加えて副交感神経（迷走神経）の活動低下にも現れており，迷走神経活動に関する種々の指標の低下が予後悪化に関係していることから，迷走神経の活動低下も単なる随伴現象ではなく病態そのものに関わっていると考えられる．

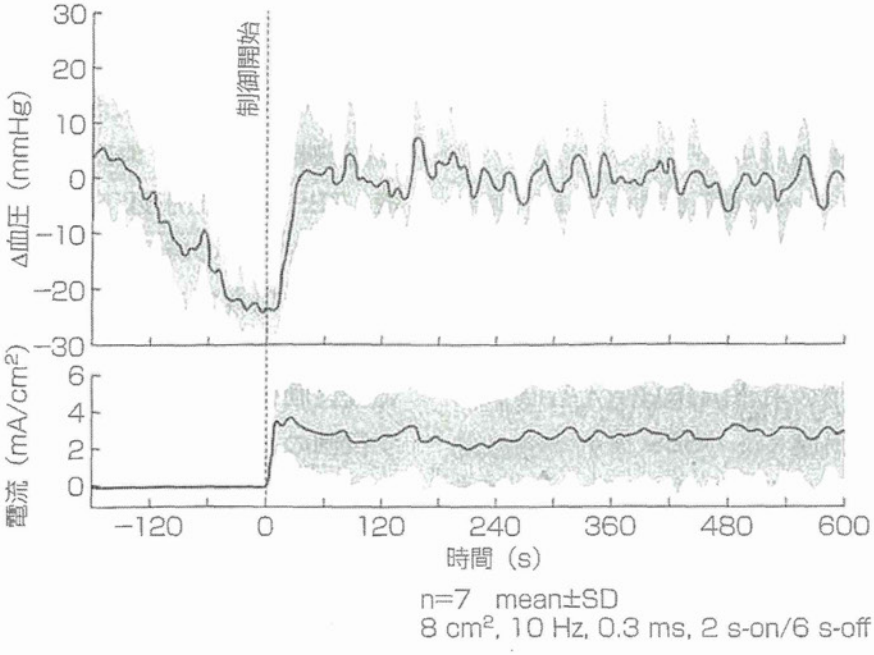

図3. 血圧安定化治療の脊髄損傷患者（経皮刺激）でのコンセプト証明

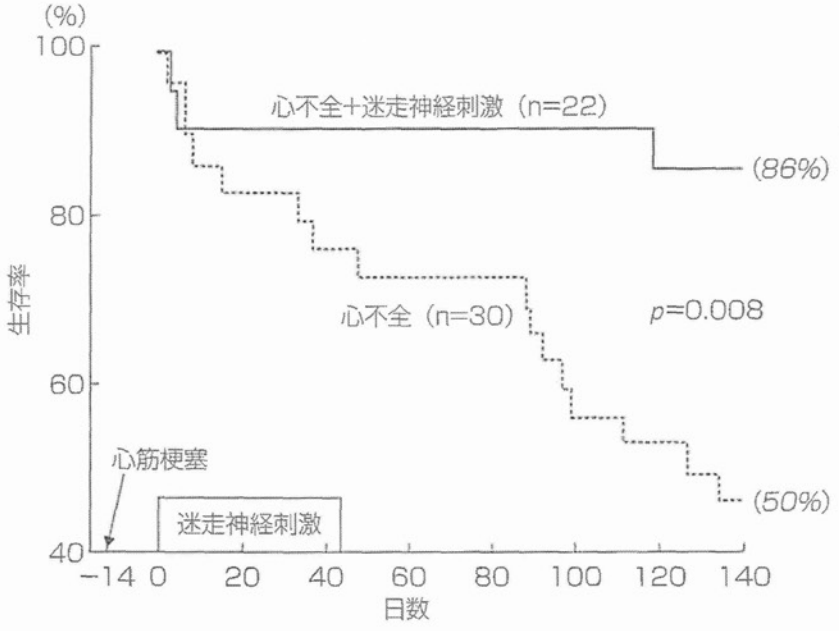

図4. 迷走神経刺激治療のラット（右頸部迷走神経刺激）でのコンセプト証明,（文献7より許可を得て引用）

しかしこれまでに迷走神経の活動低下そのものを是正する治療法はなかった.

そこで私たちは,「迷走神経の長期刺激により慢性心不全の予後を改善できる」というコンセプトのもと，迷走神経刺激治療を開発した．血圧安定化治療と異なり，生体制御自体が破綻しているため，迷走神経刺激の条件は試行錯誤で決める必要があった.

2）動物でのコンセプト証明

ラットの左冠動脈近位部を結紮して再潅流を

行わない心筋梗塞を作成した．2週間待って心筋梗塞後が完成したのち，迷走神経刺激治療の検討を行った．梗塞領域は広く（平均53%），経過とともに重症の心不全を呈した．

迷走神経刺激は右頸部迷走神経に装着した電極と外部より無線で刺激条件が制御可能な植込み型の迷走神経刺激装置により行った．別途，血圧信号を植込み装置により無線でモニタし，心拍数が約10%低下する比較的低強度の電気刺激によって迷走神経を刺激した．また刺激は間欠的（1分間の中で10秒間）に行った．

図4は6週間にわたる治療中および治療後の生存率の比較である．心不全の重症度を反映し，未治療群の生存率は治療開始後20週後には50%に留まった．しかし迷走神経刺激治療群では生存率は劇的に改善した（86%）[7]．

この改善には，長期にわたる心拍数の漸減，致死的不整脈の減少，心機能低下の軽減，心臓リモデリングの抑制，神経体液性因子（ノルエピネフリン）増加の軽減，心不全重症度を表すB型ナトリウム利尿ホルモン（BNP）増加の軽減などを伴っていた．

さらに充分量のβ遮断薬を投与しても追加的にこれらの効果があること，自発的食塩摂取量増加の軽減やバゾプレシン分泌量増加の軽減など中枢作用も示唆されること，コリンエステラーゼ阻害薬のひとつであるドネペジルによっても効果が模擬できること[8]なども明らかになってきた．

3）実用化に向けて

本治療の開発においては長期の治療が必要であり，ヒトでのコンセプト証明においても植込みデバイスによる治療が必要となる．そのため，実用化に供するのと同等の試作機を開発してその安全性を確認するのが先決となる．このように困難な点はあるが，今後企業との共同研究により実用化に向け開発を進める予定である．

おわりに

自律神経への介入治療はその即時性により機能再建に用いることができ，また薬物治療ではカバーできない病態に対して強力な効果をあげることが期待される．ペースメーカなどの電気刺激を応用した機器により実現できる治療法であるので，近い将来に実用化され広く応用される治療法になるものと考えられる．

著者のCOI（conflicts of interest）開示：本論文発表内容に関連して特に申告なし

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Central vagal activation by alpha$_2$-adrenergic stimulation is impaired in spontaneously hypertensive rats

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Abstract

Aim: To elucidate the abnormality of vagal control in spontaneously hypertensive rats (SHR) by measuring left ventricular myocardial interstitial acetylcholine (ACh) release in response to α_2-adrenergic stimulation as an index of *in vivo* vagal nerve activity.

Methods: A cardiac microdialysis technique was applied to the rat left ventricle *in vivo*, and the effect of α_2-adrenergic stimulation by medetomidine or electrical vagal nerve stimulation on myocardial interstitial ACh levels was examined in normotensive Wistar–Kyoto rats (WKY) and SHR under anaesthetized conditions.

Results: Intravenous medetomidine (0.1 mg kg^{-1}) significantly increased the ACh levels in WKY (from 2.4 ± 0.6 to 4.2 ± 1.3 nmol L^{-1}, $P < 0.05$, $n = 7$) but not in SHR (from 2.5 ± 0.7 to 2.7 ± 0.7 nmol L^{-1}, $n = 7$). In contrast, electrical vagal nerve stimulation increased the ACh levels in both WKY (from 1.0 ± 0.4 to 2.9 ± 0.9 nmol L^{-1}, $P < 0.001$, $n = 6$) and SHR (from 0.9 ± 0.2 to 2.2 ± 0.4 nmol L^{-1}, $P < 0.001$, $n = 6$). Intravenous administration of medetomidine (0.1 mg kg^{-1}) did not affect the vagal nerve stimulation–induced ACh release in either WKY or SHR.

Conclusion: Medetomidine-induced central vagal activation was impaired in SHR, whereas peripheral vagal control of ACh release was preserved. In addition to abnormal sympathetic control, vagal control by the central nervous system may be impaired in SHR.

Keywords acetylcholine, cardiac microdialysis, medetomidine, rats, vagal nerve stimulation.

While enhanced sympathetic nerve activity is one of major features of essential hypertension (Mancia *et al.* 1999), abnormality of vagal control is not fully understood, partly owing to the lack of methodology to directly assess *in vivo* vagal nerve activity. Although heart rate (HR) response under sympathetic blockade may reflect vagal nerve activity *in vivo*, several factors are included in the HR response: vagal outflow from the central nervous system, acetylcholine (ACh) release from vagal nerve terminals and responsiveness of HR to ACh. While a study by Salgado *et al.* (2007) on the bradycardic response to aortic depressor nerve stimulation suggests the reduced parasympathetic control in spontaneously hypertensive rats (SHR) compared with Wistar–Kyoto rats (WKY), more direct evidence is necessary to delineate the impairment of vagal control in SHR.

A previous study from our laboratories using anaesthetized rabbits has indicated that intravenous administration of an α_2-adrenergic agonist medetomidine activates the cardiac vagal nerve (Shimizu *et al.* 2012). In that study, a cardiac microdialysis technique was applied to monitor the changes in myocardial interstitial ACh levels in the right atrial wall near the

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sinoatrial node. We hypothesized that the medetomidine-induced vagal activation might be impaired in SHR compared to WKY. To test the hypothesis, we applied a cardiac microdialysis technique to the rat left ventricular free wall and compared the effects of intravenous administration of medetomidine on myocardial interstitial ACh levels in WKY and SHR. Furthermore, to examine whether peripheral vagal control of ACh release is impaired in SHR, myocardial interstitial ACh release in response to electrical vagal stimulation was examined in WKY and SHR.

Materials and methods

Surgical preparation

Animal care was conducted in strict accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences*, which has been approved by the Physiological Society of Japan. All protocols were reviewed and approved by the Animal Subject Committee of National Cerebral and Cardiovascular Center.

Two main protocols were conducted in male WKY (300–400 g) and SHR (320–380 g) as described in '*Protocols*' below. Each rat was anaesthetized with an intraperitoneal injection (2 mL kg^{-1}) of a mixture of urethane (250 mg mL^{-1}) and α-chloralose (40 mg mL^{-1}), and mechanically ventilated with oxygen-enriched room air. Venous catheters were inserted into bilateral external jugular veins. One venous catheter was used for continuous infusion of a 20-fold diluted solution of the above anaesthetic mixture (2–3 mL kg^{-1} h^{-1}) and Ringer's solution (5–6 mL kg^{-1} h^{-1}), and the other for the injection of test drugs. An arterial catheter was inserted into the right common carotid artery to measure the arterial pressure (AP) and HR. In Protocol 2, the vagi were sectioned at the neck, and a pair of stainless steel wire electrodes (Bioflex wire, AS633; Cooner Wire, Chatsworth, CA, USA) was attached to the distal segment of the sectioned nerve bilaterally for efferent vagal nerve stimulation. The nerves and electrodes were secured and insulated with silicone glue (Kwik-Sil; World Precision Instruments, Sarasota, FL, USA). The animal was then placed in a lateral position, and the left third to fifth ribs were partially resected to expose the heart. The pericardium was incised and a dialysis probe was implanted into the lateral free wall of the left ventricle as shown in Figure 1. Body temperature of the animal was maintained at around 38 °C by a heating pad and a lamp. At the end of the experiment, a postmortem examination confirmed that the dialysis membrane was not exposed into the left ventricular cavity.

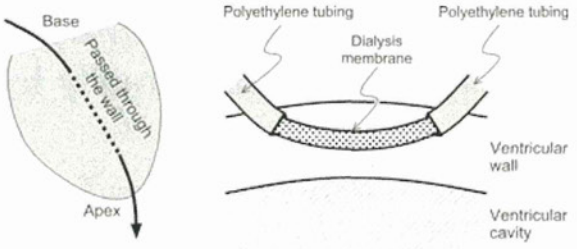

Figure 1 Schematic diagram of dialysis probe implantation. The transverse dialysis probe was implanted within the left ventricular free wall.

Dialysis technique

Dialysate concentration of ACh was measured as an index of myocardial interstitial ACh level. The materials and properties of the dialysis probe have been described previously (Akiyama *et al.* 1994). Briefly, we designed a straight, transverse dialysis probe consisting of a dialysis fibre (length, 6 mm; outer diameter, 310 μm; inner diameter, 200 μm; PAN-1200, 50 000-Da molecular weight cut-off; Asahi Chemical, Osaka, Japan) glued at both ends to polyethylene tubes (length, 25 cm; outer diameter, 500 μm; inner diameter, 200 μm). The dialysis probe was perfused with Ringer's solution containing a cholinesterase inhibitor eserine (100 μmol L^{-1}; Wako Pure Chemical, Osaka, Japan). Dialysate sampling was started from 2 h after probe implantation. In Protocol 1, the perfusion rate was set at 1 μL min^{-1}. Each sampling period was 20 min, which yielded a sample volume of 20 μL. A time lag of 10 min was allowed between actual dialysate sampling and collection of the sample into a tube, taking into account the dead space between the dialysis membrane and collecting tube. In Protocol 2 and in the supplemental protocol, to reduce possible tachyphylaxis induced by constant vagal nerve stimulation, the sampling period was halved to 10 min, while the perfusion rate was doubled to 2 μL min^{-1}. The time lag between actual dialysate sampling and sample collection was 5 min. The concentration of ACh in the dialysate was measured using a high-performance liquid chromatography system with electrochemical detection (Eicom, Kyoto, Japan) adjusted to measure low levels of ACh (Shimizu *et al.* 2009, 2012; Kawada *et al.* 2009, 2010).

Protocols

Protocol 1 ($n = 7$ each for WKY and SHR): After collecting a baseline dialysate sample for 20 min, medetomidine (0.1 mg kg^{-1}; Orion Pharma, Espoo, Finland) was injected intravenously via the jugular vein. Medetomidine initially increases AP by

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vasoconstriction via its peripheral α_2-adrenergic effect, then decreases AP as time elapses by sympathetic inhibition via its central α_2-adrenergic effect (Sinclair 2003). After allowing a 10-min stabilization period after medetomidine administration, dialysate was sampled for 20 min. Next, to avoid hypotension induced by medetomidine, intravenous infusion of an α_1-adrenergic agonist phenylephrine (250 μg kg^{-1} h^{-1}; Kowa Pharmaceuticals, Nagoya, Japan) was started simultaneous to injection of an additional dose of medetomidine (0.1 mg kg^{-1}). After allowing 10-min stabilization, dialysate was sampled for 20 min.

Protocol 2 (n = 6 for WKY and SHR each): This protocol was performed under vagotomized conditions. Before collecting a baseline dialysate sample, bilateral vagi were stimulated (20 Hz, 5 V, 2-ms pulse width) for 10 min twice with an intervening interval of 10 min in order to establish stable stimulatory conditions. A supramaximal amplitude was used in stimulation, and increasing the amplitude beyond 5 V did not induce further bradycardic response. Baseline dialysate was then sampled for 10 min. Thereafter, the vagi were stimulated for 10 min, and the corresponding dialysate sample was collected for 10 min. Next, vagal nerve stimulation was discontinued, and medetomidine (0.1 mg kg^{-1}) was injected intravenously via the jugular vein. After 10-min stabilization, dialysate was sampled for 10 min. Finally, the vagi were again stimulated for 10 min, and the corresponding dialysate sample was collected for 10 min.

Supplemental protocol (n = 5, WKY): To determine the source of ACh measured by the cardiac microdialysis, we implanted one or two dialysis probes into the left ventricular free wall in vagotomized rats. Dialysate samples were collected for 10 min before and during bilateral vagal stimulation (20 Hz, 5V, 2-ms pulse width) under control conditions. The perfusate was then replaced with the one containing 1 mmol L^{-1} of hexamethonium bromide (Wako Pure Chemical) (Akiyama *et al.* 2004). After 30-min stabilization, dialysate samples were collected before and during bilateral vagal stimulation. Finally, hexamethonium bromide was injected intravenously (60 mg mL^{-1}, bolus). After 10-min stabilization, dialysate samples were collected before and during bilateral vagal stimulation.

Statistical analysis

All data are presented as mean and SE. Data of mean HR and AP during intravenous pharmacological interventions were measured after a 10-min stabilization period. Data of mean HR and AP during vagal stimulation were measured at 5 min of vagal stimulation. In Protocol 1 and in the supplemental protocol, changes in dialysate ACh, HR and AP were analysed using repeated-measures analysis of variance (ANOVA) followed by Tukey's test for all pairwise comparisons (Glantz 2002). In Protocol 2, changes in dialysate ACh, HR and AP were analysed using repeated-measures two-way ANOVA with vagal nerve stimulation as one factor and medetomidine as the other. For ACh data, because the variance of measured ACh levels increased as the mean increased, statistical analyses were performed after logarithmic conversion (Snedecor & Cochran 1989). The AP and HR data were analysed without logarithmic conversion. Changes in ACh and HR in response to vagal nerve stimulation in WKY and SHR were compared by unpaired t-test. In all statistical analyses, differences were considered significant when $P < 0.05$.

Results

Figure 2 illustrates the results of Protocol 1. In the WKY group, medetomidine alone significantly increased myocardial interstitial ACh level from 2.4 ± 0.6 to 4.2 ± 1.3 nmol L^{-1} ($P < 0.05$) and decreased HR ($P < 0.05$) without significantly affecting AP. Medetomidine combined with phenylephrine significantly increased ACh to 5.8 ± 2.0 nmol L^{-1} ($P < 0.01$) and decreased HR ($P < 0.05$) compared to baseline levels, but did not change both parameters compared to medetomidine alone. Medetomidine combined with phenylephrine resulted in a significant elevation in AP ($P < 0.01$). In the SHR group, medetomidine alone did not affect ACh significantly (from 2.5 ± 0.7 to 2.7 ± 0.7 nmol L^{-1}), although it decreased HR ($P < 0.05$). Medetomidine alone caused significant hypotension ($P < 0.01$). Although medetomidine combined with phenylephrine prevented medetomidine-induced hypotension, it did not significantly affect the ACh level (3.2 ± 0.8 nmol L^{-1}). HR observed after administering medetomidine combined with phenylephrine was not significantly different from baseline.

Figure 3 illustrates the results of Protocol 2. Electrical vagal nerve stimulation increased the ACh levels in both WKY (from 1.0 ± 0.4 to 2.9 ± 0.9 nmol L^{-1}) and SHR (from 0.9 ± 0.2 to 2.2 ± 0.4 nmol L^{-1}) under control conditions. In the WKY group, vagal stimulation significantly increased ACh and decreased HR but did not affect AP. Medetomidine significantly decreased HR and AP but did not significantly change ACh. No significant interaction effect was detected by two-way ANOVA, indicating that medetomidine did not affect the vagal nerve stimulation–induced ACh or HR response. In the SHR group, vagal nerve stimulation significantly increased ACh and decreased HR and AP. Medetomidine significantly decreased HR and AP but

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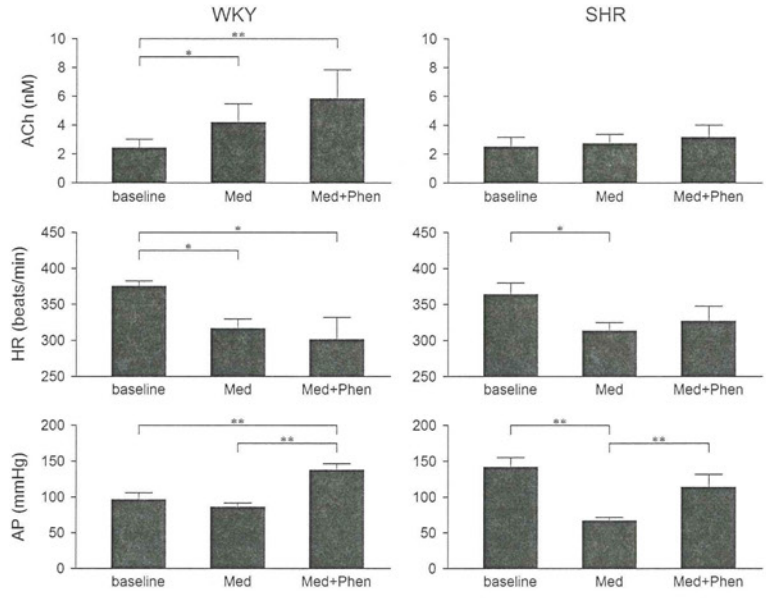

Figure 2 Effects of intravenous medetomidine (Med) alone and medetomidine combined with phenylephrine (Med+Phen) on myocardial interstitial acetylcholine (ACh) level, heart rate (HR) and arterial pressure (AP) in Wistar–Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Data are presented as mean and SE. $^{*}P < 0.05$ and $^{**}P < 0.01$ by Tukey's test after repeated-measures one-way analysis of variance (ANOVA).

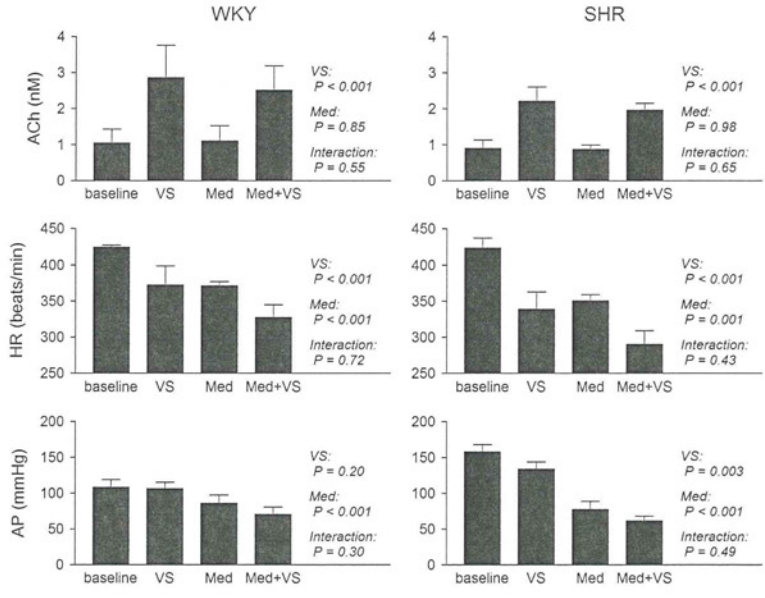

Figure 3 Effects of electrical vagal nerve stimulation (VS), intravenous medetomidine (Med) and vagal nerve stimulation with medetomidine pretreatment (Med+VS) on myocardial interstitial acetylcholine (ACh) level, heart rate (HR) and arterial pressure (AP) in Wistar–Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Data are presented as mean and SE. The *P* values represent the results of repeated-measures two-way analysis of variance (ANOVA).

did not significantly change ACh. No significant interaction effect was detected by two-way ANOVA, indicating that medetomidine did not affect the vagal nerve stimulation–induced ACh, HR or AP response. Under control conditions before medetomidine administration, the increase in ACh by vagal nerve stimulation did not differ significantly between WKY and SHR (ΔACh: 1.8 ± 0.6 vs. 1.3 ± 0.3 nmol L^{-1}, $P = 0.46$). The HR decrease induced by vagal nerve stimulation tended to be greater in SHR than in WKY (ΔHR: −51 ± 20 vs. −101 ± 12 beats min^{-1}, $P = 0.06$).

In the supplemental protocol (seven dialysis probes from five WKY rats), electrical vagal stimulation increased the myocardial interstitial ACh level from 0.7 ± 0.2 to 3.6 ± 1.2 nmol L^{-1} ($P < 0.01$) under control conditions (Fig. 4, top). Electrical vagal stimulation increased the myocardial interstitial ACh level from 0.6 ± 0.2 to 3.1 ± 1.1 nmol L^{-1} ($P < 0.01$) during the local administration of hexamethonium through the dialysis probe. There was no statistically significant difference in the vagal stimulation–induced ACh release between the control and local hexamethonium conditions. After the intravenous administration of hexamethonium, electrical vagal stimulation did not increase the myocardial interstitial ACh level (0.8 ± 0.3 to 0.7 ± 0.2 nmol L^{-1}). The intravenous administration of hexamethonium reduced prestimulation HR and abolished the vagal stimulation–induced

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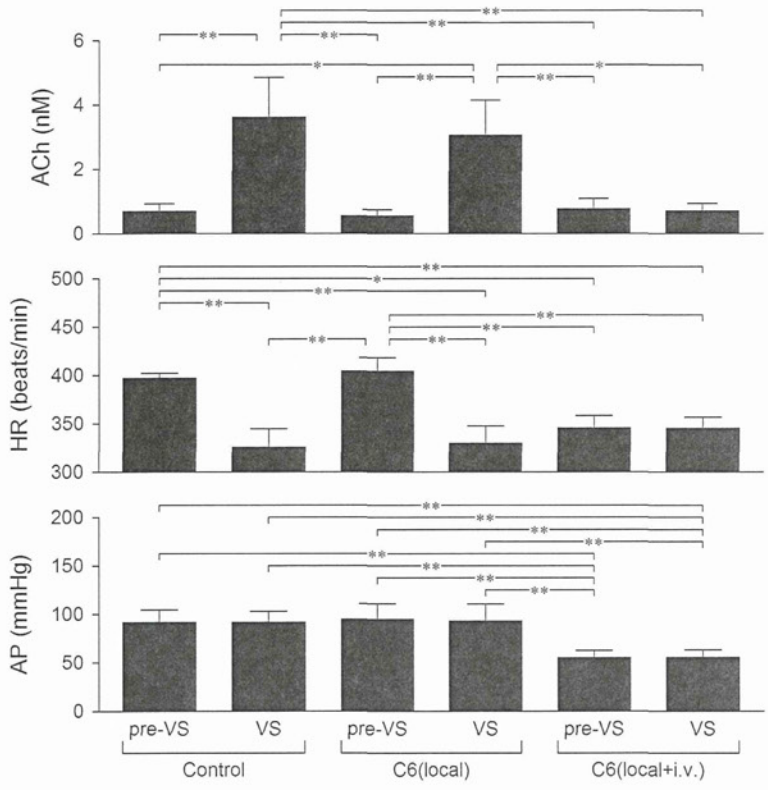

Figure 4 Effects of local administration of hexamethonium bromide [C6(local)] and intravenous administration of hexamethonium bromide [C6(local+i.v.)] on the electrical vagal stimulation–induced ACh release in five Wistar–Kyoto rats. For ACh data, seven dialysate samples from the five rats were analysed. Because intravenous administration of hexamethonium completely blocked the vagal stimulation–induced ACh release, the ACh measured by cardiac microdialysis was mainly originated from the postganglionic vagal nerves. The failure of local administration of hexamethonium to block the ACh release suggests that vagal ganglia were not present nearby the dialysis fibre. Data are presented as mean and SE. $P < 0.05$ and $P < 0.01$ by Tukey's test after repeated-measures one-way analysis of variance (ANOVA).

bradycardia (Fig. 4, middle). The intravenous administration of hexamethonium also reduced AP irrespective of vagal stimulation (Fig. 4, bottom).

Discussion

The present study demonstrated that intravenous medetomidine increased left ventricular myocardial interstitial ACh levels in WKY but not in SHR, indicating that medetomidine-induced central vagal activation was impaired in SHR. Vagal nerve stimulation increased the ACh levels in both WKY and SHR, suggesting that the peripheral vagal control of ACh release was preserved in SHR. The results of the supplemental protocol indicated that the myocardial interstitial ACh measured by cardiac microdialysis in the rat left ventricle was mainly derived from the postganglionic vagal nerve.

Effects of medetomidine on myocardial interstitial ACh release

Medetomidine is a racemic mixture of dexmedetomidine and levomedetomidine. Because levomedetomidine has been shown to be physiologically inert (Kuusela *et al.* 2000), the effects of medetomidine are mostly attributable to those of dexmedetomidine. As expected based on our previous study (Shimizu *et al.* 2012), medetomidine increased myocardial interstitial ACh level significantly in the left ventricle in WKY (Fig. 2). Because α_2-adrenergic receptors are densely distributed in the dorsal motor nucleus of the vagus and nucleus tractus solitarius (Robertson & Leslie 1985), α_2-adrenergic agonists probably activate the cardiac vagal nerve through the action on these brainstem areas. In addition, recent papers indicate that α_2-adrenergic receptor activation inhibits GABAergic neurotransmission in the nucleus ambiguus so as to increase the activity of premotor cardioinhibitory vagal neurones (Philbin *et al.* 2010), and α_1-adrenergic receptor activation facilitates GABAergic and glycinergic neurotransmission so as to reduce the activity of cardioinhibitory vagal neurones (Boychuk *et al.* 2011). In contrast to WKY, the same dose of medetomidine did not affect the ACh level in SHR, suggesting impairment of α_2-adrenergic stimulation–induced central vagal activation in SHR. Although HR was reduced by medetomidine in both WKY and SHR, no significant change in ACh level and significant hypotension observed after medetomidine injection in SHR suggest that the bradycardia observed in SHR was primarily because of sympathetic inhibition rather than vagal activation.

Intravenous medetomidine had a greater hypotensive effect in SHR than in WKY, indicating that hypertension in SHR was dependent on sympathetic

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nerve activity. The reduction in peripheral vascular resistance owing to sympathetic suppression may account for the significant hypotension induced by medetomidine in SHR. To exclude the possibility that medetomidine-induced hypotension in SHR prevented ACh release because of the deactivation of the arterial baroreflex, AP was maintained by continuous infusion of phenylephrine. The same dose of phenylephrine resulted in comparable AP elevation in both groups (WKY: $\Delta AP = 47 \pm 16$ and SHR: $\Delta AP = 52 \pm 11$ mmHg), consistent with a previous study (Jablonskis & Howe 1994). Despite the maintenance of AP above 110 mmHg in SHR, medetomidine failed to increase the ACh levels in these rats. The arterial baroreflex function is known to reset to a higher input pressure range in SHR (Nosaka & Wang 1972); therefore, the AP elevation may not have been sufficiently high to evoke a significant baroreflex-mediated vagal activation in SHR. While increasing the dose of phenylephrine could further increase AP in SHR, there is a concern of a possible confounding effect as follows. In the superfused rat atrium, α_1-adrenergic stimulation has been shown to suppress ACh outflow in response to K^+ exposure (McDonough *et al.* 1986). Such presynaptic inhibition via the α_1-adrenergic mechanism might counteract the medetomidine-induced ACh release when the dose of phenylephrine is increased. Further studies are needed to determine whether a lack of arterial baroreceptor input signal after medetomidine injection can totally explain the loss of vagal activation in SHR.

Effects of electrical vagal stimulation on myocardial interstitial ACh release

In Protocol 2, which was conducted under vagotomized conditions, medetomidine alone did not increase myocardial interstitial ACh level in WKY, suggesting that medetomidine increased the ACh level in Protocol 1 by centrally activating the cardiac vagal nerve and not by directly exciting parasympathetic ganglia or vagal nerve terminals. These results are supported by a previous study from our laboratories, in which intravenous medetomidine did not affect the vagal nerve stimulation–induced ACh release in rabbit right atrium (Shimizu *et al.* 2012). Pretreatment with medetomidine did not significantly modify the vagal nerve stimulation–induced ACh release in WKY, which agrees with previous experimental results using superfused rat atrium, in which α_1-adrenergic but not α_2-adrenergic stimulation reduced the K^+-evoked ACh outflow (McDonough *et al.* 1986).

Electrical vagal nerve stimulation induced the myocardial interstitial ACh release in SHR similar to that in WKY (Fig. 3), suggesting that peripheral vagal control of ACh release was preserved in SHR. Although *ex vivo* HR response to vagal nerve stimulation is attenuated in SHR compared to WKY in an isolated atrial/right vagus preparation (Heaton *et al.* 2007), *in vivo* HR response to vagal nerve stimulation is not different between WKY and SHR or is enhanced in SHR than in WKY depending on the age investigated (Ferrari *et al.* 1992, Minami & Head 2000, Masuda 2000). In the present study, HR response to vagal nerve stimulation tended to be enhanced in SHR, consistent with previous *in vivo* investigations. Furthermore, because medetomidine did not significantly modulate the vagal nerve stimulation–induced ACh release in SHR, the lack of ACh response to medetomidine seen in Protocol 1 may be a centrally derived defect. Loss of cardiac vagal preganglionic neurones in adult SHR may be related to the impaired central vagal activation by medetomidine (Corbett *et al.* 2007).

Vagal nerve stimulation had no significant effect on AP in WKY. An increase in the stroke volume occurs in the closed circulatory system and may compensate for the decrease in the number of heartbeat to keep cardiac output. The finding is consistent with a previous study showing that moderate vagal nerve stimulation does not significantly affect the arterial baroreflex-mediated AP regulation (Kawada *et al.* 2011). In contrast, vagal nerve stimulation decreased AP significantly in SHR irrespective of the presence of medetomidine. Because there may be no major innervation of the vagal nerve over the peripheral vasculature, the reduction in AP induced by vagal stimulation observed in SHR is likely to be cardiac origin. For instance, vagal nerve stimulation can reduce ventricular contractility in the presence of a sympathetic tone (Nakayama *et al.* 2001). Because sympathetic nerve activity is higher in SHR than in WKY (Tsunoda *et al.* 2000), vagal nerve stimulation could decrease AP by reducing ventricular contractility before the administration of medetomidine. On the other hand, because medetomidine might have suppressed sympathetic nerve activity, interaction between the vagal and sympathetic systems cannot explain the vagal nerve stimulation–induced hypotension in SHR after medetomidine administration. There is a positive force–frequency relationship (FFR) in large animals such as dogs (Mitchell *et al.* 1963, Kambayashi *et al.* 1992). In rabbits, vagal nerve stimulation decreases ventricular contractility mainly through its negative chronotropic effect, that is, via a positive FFR (Matsuura *et al.* 1997). In contrast, the FFR may be negative in the physiological HR range (200–500 beats min^{-1}) in rats (Nalivaiko *et al.* 2010). Therefore, the FFR alone might have operated to increase the ventricular contractility during bradycardia in rats. Further investigations

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