Abstract—Systemic arterial hypertension has been previously suggested to develop as a compensatory condition when central nervous perfusion/oxygenation is compromised. Principal sympathoexcitatory C1 neurons of the rostral ventrolateral medulla oblongata (whose activation increases sympathetic drive and arterial blood pressure) are highly sensitive to hypoxia, but the mechanisms of this O₂ sensitivity remain unknown. Here, we investigated potential mechanisms linking brainstem hypoxia and high systemic arterial blood pressure in the spontaneously hypertensive rat. Brainstem parenchymal PO₂ in the spontaneously hypertensive rat was found to be ≈15 mmHg lower than in the normotensive Wistar rat at the same level of arterial oxygenation and systemic arterial blood pressure. Hypoxia-induced activation of rostral ventrolateral medulla oblongata neurons was suppressed in the presence of either an ATP receptor antagonist MRS2179 or a glycogenolysis inhibitor 1,4-dideoxy-1,4-imino-d-arabinitol, suggesting that sensitivity of these neurons to low PO₂ is mediated by actions of extracellular ATP and lactate. Brainstem hypoxia triggers release of lactate and ATP which produce excitation of C1 neurons in vitro and increases sympathetic nerve activity and arterial blood pressure in vivo. Facilitated breakdown of extracellular ATP in the rostral ventrolateral medulla oblongata by virally-driven overexpression of a potent ectonucleotidase transmembrane prostatic acid phosphatase results in a significant reduction in the arterial blood pressure in the spontaneously hypertensive rats (but not in normotensive animals). These results suggest that in the spontaneously hypertensive rat, lower PO₂ of brainstem parenchyma may be associated with higher levels of ambient ATP and t-lactate within the presynaptic circuits, leading to increased central sympathetic drive and concomitant sustained increases in systemic arterial blood pressure.

Key Words: adenosine triphosphate ■ hypertension ■ hypoxia ■ lactic acid ■ sympathetic nervous system

Hypertension is one of the main risk factors for the development of many cardiovascular diseases. Despite significant progress in the diagnosis and treatment of hypertension, approximately only half of patients show satisfactory response to treatment. This poor efficacy might be because of the fact that conventional antihypertensive therapies are aimed at downstream peripheral mechanisms which maintain high systemic arterial blood pressure, while primary factors responsible for the development of the condition remain untreated.

The pathophysiology of systemic arterial hypertension is complex and in general poorly understood, but over the last 3 decades studies in animal models and patients with hypertension have provided significant evidence that activation of the sympathetic nervous system is linked to the development and maintenance of the condition. Vasomotor and cardiac activities of spinal sympathetic preganglionic neurons depend on tonic descending excitatory drive generated by sympathoexcitatory (presympathetic) neuronal networks residing in the hypothalamus and the brainstem: the rostral ventrolateral medulla (RVLM), rostral ventromedial and midline medulla, the A5 cell group of the pons, and the paraventricular hypothalamic nucleus. Bulbospinal neurons of the RVLM which belong to the catecholaminergic C1 group are believed to be of a prime importance for the maintenance of vasomotor sympathetic tone.

One of the potential mechanisms which may be responsible for sympathetic activation in hypertension is based on the operation of a so-called Cushing response characterized by a triad of high blood pressure, irregular breathing, and bradycardia.
Although originally described as an autoregulation mechanism triggered under extreme pathological conditions such as brain ischemia, it is currently viewed as a physiological compensatory response to compromised brain perfusion or brain hypoxia. Brainstem vasculature of patients with hypertension and of animal models of hypertension (e.g., spontaneously hypertensive rat [SHR]) is considerably narrower (compared with the respective normotensive counterparts), resulting in high cerebral artery resistance. This is not a consequence of hypertension as it occurs prior to its development, at least in the SHR. Thus, the neuronal sympathoexcitatory networks that control the arterial blood pressure have been suggested to be hypoperfused/hypoxic. By increasing systemic arterial blood pressure in response to compromised brainstem perfusion, the Cushing mechanism would be expected to produce changes in the circulatory system to preserve oxygen delivery and maintain brain oxygenation at the expense of systemic hypertension.

The mechanisms underlying sympathetic activation associated with compromised brain tissue perfusion and hypoxia remain unknown. Presympathetic RVLM neurons are highly sensitive to hypoxia, but the mechanisms of their sensitivity have never been addressed. Brain tissue hypoxia is expected to be associated with increases in the level of extracellular lactate, which was recently shown to have a profound excitatory effect on another notable population of brainstem catecholaminergic neurons residing in the pontine locus coeruleus. In addition, we have previously shown that hypoxia triggers release of ATP within the RVLM. Other pharmacological studies have revealed that activation of ATP receptors in the RVLM by microinjections of ATP or stable ATP analogues increases the excitability of C1 neurons and leads to the increases in the arterial blood pressure, heart rate, and renal sympathetic nerve activity.

This study was designed to test the hypothesis that brainstem hypoxia is associated with the pathogenesis of systemic arterial hypertension. We measured oxygen tension from within the presympathetic RVLM region of the brainstem in the SHRs and control Wistar rats, determined whether oxygen sensitivity of presympathetic C1 neurons is direct or mediated by prior release and actions of ATP and lactate, evaluated the effects of l-lactate on sympathetic nerve activity and the arterial blood pressure, and determined the effect of blocking ATP-mediated signaling in the RVLM on systemic arterial blood pressure in the SHRs and their normotensive counterparts.

**Methods**

All animal experiments were performed in accordance with the European Commission Directive 86/609/EEC (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes) and the UK Home Office (Scientific Procedures) Act (1986) with project approval from the respective Institutional Animal Care and Use Committees. Detailed description of the Materials and Methods used in the current study is available in the online-only Data Supplement.

**Results**

**Brainstem of the SHR Is Hypoxic at Normal Levels of the Arterial Blood Pressure**

At baseline conditions, ventrolateral medullary PO₂ in anesthetized SHRs was found to be slightly lower compared with their age- and sex-matched normotensive Wistar rats (18±3 mm Hg [n=5] versus 26±4 mm Hg [n=6]; P=0.07; Figure 1B). When the arterial blood pressure of anesthetized SHRs was lowered to the level of normotensive Wistar rats (from 144±3 mm Hg to 93±1 mm Hg) by intravenous infusion of sodium nitroprusside (arterial PO₂ level was kept at 100 mm Hg), brainstem PO₂ decreased to 11±1 mm Hg (P=0.006, paired t test, RVLM PO₂ in the SHRs at baseline versus in normotensive conditions; Figure 1B). When mean arterial blood pressure of Wistar rats was increased to the level of SHRs (from 97±1 mm Hg to 145±5 mm Hg) by intravenous infusion of vasopressin (while keeping arterial PO₂ level at 100 mm Hg), brainstem PO₂ increased to 35±3 mm Hg (P=0.019, paired t test, RVLM PO₂ in Wistar rats at baseline versus in hypertensive conditions; Figure 1B). Thus, resting RVLM PO₂ in SHRs is lower than that of hypertensive Wistar rats (P=0.006, unpaired t test; Figure 1B), and resting RVLM PO₂ in Wistar rats is higher than that of normotensive SHRs (P=0.025, unpaired t test; Figure 1B). Similar data were obtained when tissue PO₂ measurements were taken from within the dorsal medullary nucleus of the solitary tract. Baseline PO₂ in the

**Figure 1.** Brainstem of the spontaneously hypertensive rat (SHR) is hypoxic at physiological levels of the systemic arterial blood pressure. A, Schematic drawing of the rat brain in sagittal projection illustrating the site of PO₂ measurements taken from the anatomic location of the presympathetic circuits of the rostral ventrolateral medulla (RVLM). B, Summary data showing parenchymal PO₂ levels in the RVLM of anesthetized SHRs and Wistar rats. PO₂ measurements were taken before and during intravenous infusion of sodium nitroprusside (SNP) and vasopressin (VP) in SHRs and Wistar rats, respectively. Data are presented as means±SEM. Note that because the arterial blood pressure in these experiments was clamped, SE values are small and the respective error bars are embedded within the symbols. Paired t test: RVLM PO₂ in Wistar rats, resting vs vasopressin (P=0.019); RVLM PO₂ in SHRs, resting vs SNP (P=0.006). Unpaired t test: RVLM PO₂ in SHRs vs hypertensive Wistar rats (P=0.006); RVLM PO₂ in normotensive Wistar rats vs normotensive SHRs (P=0.025).
nucleus of the solitary tract of anesthetized SHRs (n=3) was
16±2 mm Hg decreasing to 8±3 mm Hg (P<0.05, paired t
test) when the arterial blood pressure was lowered to the normo-
tensive level. In age- and sex-matched normotensive Wistar
rats (n=3), baseline nucleus of the solitary tract PO2 was 19±3
mm Hg (n=3) increasing to 30±3 mm Hg (P<0.05, paired t
test) when blood pressure was increased to the level recorded
in the SHRs. These data confirm that the level of the arterial
blood pressure determines PO2 of the brainstem parenchyma.
The data also demonstrate that the brainstem PO2 in the SHR
is ≈15 mm Hg lower than in the Wistar rat at the same level of
the systemic arterial blood pressure.

Figure 2. Release and actions of ATP and lactate mediate activation of rostral ventrolateral medulla (RVLM) neurons during hypoxia. A, Summary data obtained in vitro using horizontal slices of the rat brainstem showing tonic release of lactate from the ventral surface of
the medulla oblongata and peak lactate release during hypoxia. Inset, Schematic drawing of a horizontal brainstem slice illustrating dual
recording configuration of lactate and null (control) biosensors placed on the ventral medullary surface. Difference in current between
lactate and null biosensors was used to determine the amount of lactate release. py, pyramidal tract; XII hypoglossal rootlets. B, Putative
presympathetic C1 RVLM neurons visualized in organotypic brainstem slices after transduction with an adenoviral vector to express
generically encoded Ca2+ indicator TN-XXL under the control of PRSx8 promoter. C, Raw traces (changes in intracellular [Ca2+] of 2
individual neurons are shown on each plot) illustrating robust and reproducible responses of the RVLM neurons to hypoxia (upper plot)
as well as the effects of ATP receptor antagonist MRS2179 (30 µmol/L) (middle) and glycogenolysis inhibitor 1,4-dideoxy-1,4-imino-
D-arabinitol (DAB) (500 µmol/L) (bottom) on hypoxia-induced [Ca2+] responses of these neurons (ratiometric imaging using TN-XXL).
D, Summary data illustrating the effects of MRS2179 and DAB on hypoxia-induced [Ca2+] responses of putative C1 neurons. Data are
presented as means±SEM.
ATP and Lactate Mediate Excitation of RVLM Neurons at Low PO\textsubscript{2}

We next determined whether the sensitivity of RVLM neurons to hypoxia is direct or mediated by prior release and actions of ATP or lactate or both. In the acute brainstem slices of adult Wistar rats, hypoxia was associated with facilitated release of lactate (Figure 2A). Lactate biosensors placed in a direct contact with the ventral surface of the medulla oblongata detected tonic release of lactate of 396±58 µmol/L increasing to 507±71 µmol/L during 4 minutes of hypoxia (n=10; \( P<0.001\), paired \( t \) test; Figure 2A). We demonstrated previously that release of ATP in the brainstem is also facilitated during hypoxia,\textsuperscript{24} and the next experiment examined whether the sensitivity of RVLM neurons to low PO\textsubscript{2} is dependent on ATP- and lactate-mediated signaling.

Hypoxia-induced [Ca\textsuperscript{2+}] responses in putative C1 neurons were visualized in organotypic brainstem slices using the genetically encoded Ca\textsuperscript{2+} indicator TN-XXL expressed under the control of the PRSx8 promoter (Figure 2B). Confirming previously reported data,\textsuperscript{20,21} hypoxia triggered robust and reproducible [Ca\textsuperscript{2+}] elevations in 100% of the recorded neurons (Figure 2C). Hypoxia-induced [Ca\textsuperscript{2+}], responses of these putative C1 neurons were markedly reduced in the presence of an ATP receptor antagonist MRS2179 (30 µmol/L; n=19, \( P=0.005\), paired \( t \) test; Figure 2C and 2D) or after incubation of the slice with glycogenolysis inhibitor 1,4-dideoxy-1,4-imino-d-arabinitol (500 µmol/L; n=13, \( P=0.005\), paired \( t \) test; Figure 2C and 2D). These data suggest that oxygen sensitivity of RVLM neurons is not intrinsic but indirect, and mediated by actions of ATP and lactate released from as yet unknown cellular source, but most likely astrocytes.\textsuperscript{23,28,29}

Figure 3. L-lactate activates rostral ventrolateral medulla (RVLM) neurons in vitro and induces increases in the sympathetic nerve activity and the arterial blood pressure in vivo. A, Putative C1 neurons in organotypic brainstem slice culture transduced to express enhanced green fluorescent protein under the control of PRSx8 promoter and visualized for patch clamp recordings. B, Representative trace of the membrane potential changes and electrical activity of a putative C1 neuron showing depolarization and increased rate of action potential firing in response to application of L-lactate (2 mmol/L, pH 7.4). C, Representative trace and summary data (right) illustrating [Ca\textsuperscript{2+}] responses of putative C1 neurons to application of L-lactate (2 mmol/L, pH 7.4) and KCl (20 mmol/L) (ratiometric imaging using TN-XXL). D, Raw data obtained in anesthetized and artificially ventilated rat showing changes in heart rate, arterial blood pressure, and sympathetic nerve activity induced by L-lactate (20 mmol/L, pH 7.4) applied on the ventral surface of the medulla oblongata. E, Summary data illustrating the effect of L-lactate applied on the ventral surface of the medulla oblongata on systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR) and renal sympathetic nerve activity (RSNA). Data are presented as means±SEM. BP indicates arterial blood pressure.
l-Lactate Activates RVLM Neurons In Vitro and Induces Increases in the Sympathetic Nerve Activity and the Arterial Blood Pressure In Vivo

Putative C1 neurons recorded in organotypic brainstem slices (Figure 3A) responded to bath application of l-lactate (2 mmol/L, pH 7.4) with sustained depolarization ($V_m$ increased from $-62\pm5$ mV to $-56\pm5$ mV, $n=4$, $P=0.006$, paired t test) and increased rate of action potential firing (Figure 3B). l-lactate (2 mmol/L) also triggered robust [Ca$^{2+}$]i elevations in ≈60% (8 of 12 neurons that responded to KCl, 20 mmol/L) of the recorded putative C1 neurons expressing TN-XXL (Figure 3C). Lactate-induced [Ca$^{2+}$]i elevations in RVLM neurons were remarkably strong (average peak of the response reaching 80% of that triggered by KCl) with a complete recovery within ≈5 minutes of lactate washout (Figure 3C). In pentobarbital-anesthetized and artificially ventilated normotensive Wistar rats (end-tidal CO$_2$ was kept at ≈3.5±0.5% throughout the experiments), application of l-lactate (20 mmol/L, 30 µL) on the ventral surface of the medulla oblongata induced profound and sustained (lasting for at least 10 minutes) sympathoexcitatory response, characterized by significant increases in renal sympathetic nerve activity (by 66±18%, $P=0.009$, paired t test), systolic arterial blood pressure (from 106±2 to 131±8 mmHg, $P=0.002$, paired t test), diastolic arterial blood pressure (from 52±5 to 68±4 mmHg, $P=0.002$, paired t test), and heart rate (from 376±26 to 398±24 beats min$^{-1}$, $P=0.001$, paired t test; Figure 3D and 3E). These data are consistent with recent observations showing that central catecholaminergic neurons are highly sensitive to lactate$^{23}$ and suggest that higher levels of ambient lactate in the RVLM may contribute to the increased activity of presympathetic C1 bulbospinal

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**Figure 4.** Transmembrane prostate acidic phosphatase (TMPAP) expression in the rostral ventrolateral medulla (RVLM) reduces the degree of hypertension in the spontaneously hypertensive rat (SHR). **A.** TMPAP–enhanced green fluorescent protein (EGFP) expression in the C1 region of the RVLM (TH, tyrosine hydroxylase). Bottom image is a high magnification micrograph showing a proportion of TH-positive neurons expressing TMPAP-EGFP. **B.** Schematic drawing of the rat brainstem in a series of coronal projections illustrating the representative extent of TMPAP expression in relation to the anatomic location of the RVLM presympathetic circuits. Numbers indicate distance from Bregma. TMPAP expression was highest in the ventrolateral medullary regions located at −11.80 mm from Bregma. **C.** Summary data showing that bilateral expression of TMPAP-EGFP within the C1 region of the RVLM results in a significant reduction of the arterial blood pressure in the SHR. The effect was sustained for 3 weeks. Note that TMPAP expression in the RVLM had no effect on the arterial blood pressure in Wistar rats. Data are presented as means±SEM. *Significant difference between SHRs expressing EGFP and SHRs expressing TMPAP-EGFP in the RVLM ($P<0.05$).
vasomotor neurons and concomitant sympathetic activation and hypertension observed in the SHR.

**Facilitated Breakdown of Extracellular ATP in the RVLM Reduces the Degree of Hypertension in the SHR**

In the SHR, overexpression of a potent ectonucleotidase, transmembrane prostate acidic phosphatase (TMPAP), within the C1 area of the RVLM (Figure 4A and 4B; to promote facilitated ATP breakdown) was associated with a significant reduction in systemic arterial blood pressure (Figure 4C). This effect was maintained for 3 weeks of TMPAP expression in the RVLM [mean arterial blood pressure was 116±9 mm Hg (n=7) in the SHRs expressing TMPAP in the RVLM versus 153±8 mm Hg (n=9) in the SHRs expressing enhanced green fluorescent protein; \( P=0.009 \), Kruskal–Wallis ANOVA by ranks]. There were no differences in mean arterial blood pressure between 2 groups of SHR 5 weeks after the injections of TMPAP or enhanced green fluorescent protein–expressing viral vectors (Figure 4C). TMPAP expression and activity in the RVLM had no effect on the arterial blood pressure of Wistar rats [TMPAP: 91±4 mm Hg (n=8) versus enhanced green fluorescent protein: 93±4 mm Hg (n=8); \( P=0.836 \), Kruskal–Wallis ANOVA by ranks]. Immunohistochemical analysis identified a proportion of tyrosine hydroxylase–positive (C1) RVLM neurons expressing TMPAP (Figure 4A) and a general strong expression of the transgene along the rostro-caudal extent of the RVLM (between \(-11.60 \) and \(-12.80 \) mm from Bregma; Figure 4B). Thus, TMPAP expression and facilitated breakdown of ATP in the RVLM lowers arterial blood pressure in the SHR without affecting this hemodynamic variable in control animals, suggesting that ATP-mediated signaling contribute to the maintenance of systemic arterial hypertension in this model.

**Discussion**

This study tested the hypothesis that brainstem hypoxia contributes to the pathogenesis of systemic arterial hypertension. This hypothesis is difficult to test directly as this would require an experimental paradigm allowing differential precise manipulation of peripheral and cerebral oxygenation at different stages of development, including young prehypertensive SHRs. However, our evidence strongly supports the hypothesis. We found that the brainstem of the adult SHR is hypoxic (\( \text{PO}_2 \approx 10 \) mm Hg) at physiological levels of the arterial \( \text{PO}_2 \) (100 mm Hg) and systemic arterial blood pressure (mean \( \approx 95 \) mm Hg). \( \text{PO}_2 \) measurements were taken from within the brainstem region that contains a major group of RVLM bulbospinal sympathoexcitatory (presympathetic) C1 neurons which generate vasomotor sympathetic tone.\(^7,12,30\) These neurons are known to be excited by low \( \text{PO}_2 \)\(^2,20,21,31\). Our data indicate that their sensitivity to hypoxia is (at least partially) indirect (ie, nonintrinsic) and mediated by prior release and actions of ATP and lactate. Tissue hypoxia always leads to lactate release/accumulation\(^3\) (we confirm this in the current study, see Figure 2A), and here we demonstrate that lactate is capable of increasing the activity of RVLM neurons. We also found that application of lactate to the ventral surface of the brainstem in normotensive animals produces profound sympathoexcitation and increases in the arterial blood pressure. Hypoxia-evoked release of ATP and ATP stimulatory actions on C1 neurons, central sympathetic drive, and systemic arterial blood pressure have been demonstrated previously.\(^24,25,27\) Finally, in the SHR, blockade of ATP-mediated signaling in the RVLM presympathetic area by virally-driven overexpression of a potent ectonucleotidase (TMPAP), resulted in a significant lowering of the arterial blood pressure—the effect which was sustained for 3 weeks. The aim of this experiment was to achieve widespread and strong transgene expression in the general RVLM area. This would ensure effective ATP breakdown and blockade of ATP-mediated paracrine (volume) signaling. Although, only a proportion of the RVLM C1 neurons was transduced, expression of TMPAP by other cellular elements in the area was clearly sufficient to achieve significant reduction of the arterial blood pressure in the SHR. Together these data suggest that decreased parenchymal \( \text{PO}_2 \) in the RVLM leads to higher levels of ambient ATP and lactate which increase the excitability of RVLM neurons, leading to enhanced vasomotor sympathetic tone and high systemic arterial blood pressure. We propose that in the SHR this serves as a compensatory condition needed to maintain adequate blood supply and oxygenation of the brain.

Several previous studies have shown that cerebral vascular resistance is high in patients with hypertension as well as in animal models of hypertension, including the SHRs.\(^14-17,33\) In rats, increasing cerebral vascular resistance was shown to raise sympathetic activity and the arterial blood pressure.\(^16\) Moreover, in the SHR, development of hypertension is associated with progressive impairment of neurovascular coupling, elevated baseline capillary red blood cell velocity,\(^34\) and impaired autoregulation because of vascular hypertrophy.\(^35\) Our data are consistent with these observations and directly demonstrate that the brainstem of SHRs becomes hypoxic when systemic arterial blood pressure decreases to physiological levels.

Histological analysis revealed robust TMPAP expression in the RVLM even at 6 weeks after the delivery of viral vectors (not shown). Therefore, fading TMPAP expression and declining activity are unlikely to be responsible for the recovery of the hypertensive phenotype in the SHR. Although TMPAP activity is effective in reducing systemic arterial blood pressure, our oxygen measurements suggest that this would also result in reduced cerebral oxygenation. We hypothesize that compensation is likely to develop over time to restore high arterial blood pressure by either recruitment of other mechanisms driving the activity of RVLM presympathetic neurons or by enhanced activity of presympathetic neurons residing in other regions of the central nervous system, not targeted to express TMPAP (eg, paraventricular nucleus). Moreover, at later stages of
hypertension development in this model, other factors (renal and vascular) may become more significant in maintenance of the condition.

Earlier in vivo and in vitro studies have demonstrated that presympathetic C1 neurons are highly sensitive to hypoxia (or chemical hypoxia induced by CN− application)\textsuperscript{20,21,36–38}. Confirming these reports, hypoxia was found to trigger robust and reproducible [Ca\textsuperscript{2+}]i elevations in 100% of the recorded RVLM neurons. Hypoxia-induced responses in these putative C1 neurons were reduced to a similar extent either in the presence of an ATP receptor antagonist MRS2179 (which is acting preferentially at metabotropic P2Y\textsubscript{1} receptors) or after incubation of the slice with glycogenolysis inhibitor 1,4-dideoxy-1,4-imino-D-arabinitol. These data suggest that sensitivity of C1 neurons to decreases in PO\textsubscript{2} is mostly indirect and mediated by prior release and actions of ATP and lactate. In this study, we demonstrate directly that hypoxia leads to a significant increase in lactate efflux detected at the ventral surface of the brainstem, whereas our previous study reported profound hypoxia-induced release of ATP in the medulla oblongata—an effect observed in anesthetized and artificially ventilated rats and in acute brainstem slices in vitro.\textsuperscript{24}

C1 neurons are known to be sensitive to ATP. ATP or stable ATP analogues increase their activity, triggers sympatheoexcitation, and increases arterial blood pressure in normotensive rats.\textsuperscript{25,27,39} A role of purinergic signaling in modulating the activity of RVLM presympathetic circuits is also supported by a more recent evidence showing that P2Y\textsubscript{1} receptor-mediated activation of C1 neurons contributes to sympathetic and blood pressure responses elicited by activation of the peripheral chemoreceptors.\textsuperscript{40} Furthermore, ATP-mediated signaling may also contribute to alterations in the central nervous mechanisms of autonomic control and development of hypertension in rats subjected to chronic intermittent hypoxia. This animal model shows significantly increased expression of ionotropic ATP receptors (P2X\textsubscript{2} and P2X\textsubscript{3} subunits in particular) in the RVLM\textsuperscript{41} and markedly enhanced sympathetic responses to microinjections of ATP into the RVLM.\textsuperscript{42}

Responses of the RVLM neurons to lactate application were found to be similar in magnitude and time course to those observed recently in neurons of the pontine locus coeruleus, a major cluster of catecholaminergic cells in the central nervous system.\textsuperscript{21} Lactate is produced by activated astroglial cells and stimulates neurons of the locus coeruleus to release norepinephrine, and these effects are mimicked by application of exogenous lactate.\textsuperscript{23} As expected, hypoxia triggered release of lactate in the ventrolateral medulla (Figure 2A), and lactate produced potent sympathoexcitatory responses in vivo (Figure 3). We propose that interfering with lactate-mediated signaling could be a potential therapeutic strategy that may help to attenuate pathological sympatheoexcitation associated with systemic arterial hypertension; however, because of current unavailability of a suitable blocker of lactate actions, we were not able to test this hypothesis in this study.

Nonexcitable cells in the brain (e.g., astrocytes) communicate predominantly via release of ATP.\textsuperscript{28,29,43} Astrocytes are also the only type of brain cell which store glycogen\textsuperscript{44} and according to the lactate shuttle hypothesis\textsuperscript{45} supply energy to neurons in the form of lactate. Although the identity of cells which release ATP and lactate responsible for hypoxia-induced activation of RVLM neurons remains unknown, astroglia represents a plausible source. Astrocytes can detect various sensory modalities such as changes in brain parenchymal pH and glucose levels.\textsuperscript{26,46} C1 neurons are activated when RVLM astrocytes are selectively stimulated using an optogenetic approach.\textsuperscript{43} These astrocyte-driven responses of C1 neurons were found to be abolished in the presence of an ATP-degrading enzyme apyrase, suggesting that ATP mediates communication between astrocytes and these presympathetic neurons.\textsuperscript{43} Furthermore, in vivo experiments conducted in anesthetized and artificially ventilated rats showed that optogenetic stimulation of RVLM astrocytes increases sympathetic nerve activity and systemic arterial blood pressure.\textsuperscript{43}

A major clinical problem associated with hypertension is stroke. Many strokes develop as a consequence of brain ischemia rather than hemorrhage. It is of interest that there is a significant association between hypertension before and after posterior cerebral artery infarction compared with anterior cerebral artery infarction.\textsuperscript{17} These observations in humans further support the hypothesis that brainstem hypoperfusion/hypoxia is an important contributor to the development of hypertension. Whether this is also the case in other cardiovascular diseases where central sympathetic drive is high is not known. However, we have recently shown that a similar mechanism involving facilitated ATP-mediated signaling in the RVLM contributes to sympathetic activation during the development of myocardial infarction-induced heart failure.\textsuperscript{43} Interestingly, a recent study using cerebral oximetry has demonstrated that cerebral tissue oxygen saturation in many heart failure patients is low, despite nearly normal levels measured in the arterial blood.\textsuperscript{37} It seems that the same mechanism activated by brainstem hypoxia might be responsible for heightened central sympathetic tone in both heart failure and systemic arterial hypertension.

**Perspectives**

The data obtained in the present study suggest that in the SHR low parenchymal PO\textsubscript{2} results in higher levels of ambient ATP and lactate within the presympathetic areas of the brainstem leading to the increased activity of sympathoexcitatory neurons and concomitant sustained increases in the arterial blood pressure.

**Sources of Funding**

Experimental work described in this paper was funded by The Wellcome Trust (UK) and the British Heart Foundation. N. Marina is a British Heart Foundation Intermediate Basic Research Fellow (ref. FS/13/5/29927). A.V. Gourine is a Wellcome Trust Senior Research Fellow (ref. 095064).

**Disclosures**

None.
References


**What Is New?**

- Brainstem parenchymal oxygen tension in the spontaneously hypertensive rat is ≈15 mm Hg lower than in the normotensive Wistar rat at the same level of arterial oxygenation and systemic arterial blood pressure.
- Hypoxia-induced activation of presympathetic brainstem circuits is mediated by actions of ATP and lactate released in low $P_{O_2}$ conditions.
- Sustained blockade of ATP-mediated signaling within the presympathetic brainstem circuits results in a significant decrease in systemic arterial blood pressure in the spontaneously hypertensive rat (but not in normotensive animals).
- These findings are the first of its kind to provide a fundamental insight into the mechanisms that link central nervous system hypoxia, increased sympathetic activity, and high systemic arterial blood pressure.

**What Is Relevant?**

- Brainstem hypoxia contributes to the development of systemic arterial hypertension.
- Signaling pathways activated by central hypoxia represent a potential therapeutic target for the treatment of systemic arterial hypertension.

**Summary**

Low $P_{O_2}$ of the brainstem parenchyma may be associated with higher levels of ambient ATP and lactate within the presympathetic circuits, leading to increased central sympathetic drive and concomitant sustained increases in the arterial blood pressure.
Brainstem Hypoxia Contributes to the Development of Hypertension in the Spontaneously Hypertensive Rat


Hypertension. 2015;65:775-783; originally published online February 23, 2015;
doi: 10.1161/HYPERTENSIONAHA.114.04683
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Brainstem hypoxia contributes to the development of hypertension in the spontaneously hypertensive rat

by

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Expanded Materials and Methods

All animal experimentations were performed in accordance with the European Commission Directive 86/609/EEC (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes) and the UK Home Office (Scientific Procedures) Act (1986) with project approval from the respective Institutional Animal Care and Use Committees.

Measurements of brainstem parenchymal $PO_2$ in vivo

Measurements of the arterial blood pressure were first taken in awake animals prior to the recordings of brainstem parenchymal $PO_2$ which were performed under anesthesia. Since brainstem $PO_2$ is dependent on systemic arterial blood pressure, recordings taken in each individual awake animal were used to maintain arterial blood pressure at exactly the same level while the animal was anesthetized for the brainstem $PO_2$ measurements.

Non-invasive arterial blood pressure measurements were performed in awake male SHRs (14 weeks of age, n=5) and age- and sex-matched normotensive Wistar rats (n=6) by the tail cuff plethysmography method. Blood pressure measurements were performed by the same investigator and at the same time of the day for all the rats. Briefly, the rats were placed on a heated platform at 37°C in a temperature-controlled (~30°C) quiet area. The tail was instrumented with a cuff and a volume pressure recording sensor (CODA system, Kent Scientific, Torrington, USA) and a total of 20-30 measurements were taken over a period of 15 min. All conscious
animals used in this study were acclimatized to the recording environment with 10 control blood pressure measurements at the beginning of each recording session. At least 10 additional measurements were subsequently taken and recorded values were averaged to determine the resting level of the arterial blood pressure.

The animals were subsequently anesthetized with 5% isoflurane in air and were left to breathe spontaneously. Anesthesia was maintained with 2-2.5% isoflurane and body temperature was maintained at ~37.0°C using a servo-controlled heating blanket. The femoral artery and vein were cannulated for monitoring of the arterial blood pressure and administration of drugs, respectively. Resting arterial blood pressure was kept at the level recorded during wakefulness (144±3 mmHg in the SHRs and 97±1 mmHg in Wistar rats) by adjusting the depth of anesthesia. The animal was placed in a stereotaxic frame and a small hole was drilled in the occipital bone. \( PO_2 \) in the brain parenchyma was measured using a fluorescence detection technique. An Oxylite™ optical oxygen sensor (Oxford Optronix, Oxford, UK) was inserted into the RVLM using the following coordinates from Bregma: 11.5 mm posterior, 2 mm lateral and 8.7 mm ventral (Fig 1A) (1;2). In a separate series of experiments \( PO_2 \) measurements were also taken from the dorsal medulla oblongata within the anatomical region of the nucleus of the solitary tract (at the level of \textit{calamus scriptorius}, 1.5 mm lateral to the midline and 0.6 mm deep from the dorsal brainstem surface). Upon completion of all surgical procedures (including placement of the oxygen sensing probe) the animals were allowed to stabilize for 30 min. During this period, the depth of anesthesia was monitored using stability of blood pressure, and the absence of corneal reflexes and flexor responses to a paw-pin. Arterial \( PO_2 \) was monitored and kept at 100±3 mmHg by adjusting the level of
supplemental O₂. Measurements of baseline brainstem PO₂ were taken at resting conditions and following pharmacological manipulation of blood pressure levels. Intravenous infusion of sodium nitroprusside or vasopressin (adjusted doses) were used to lower the arterial blood pressure of anesthetized SHRs to the level of normotensive Wistar rats (from 144±3 mmHg to 93±1 mmHg) and to increase arterial blood pressure of Wistar rats to the level recorded in the SHRs (from 97±1 mmHg to 145±5 mmHg), respectively.

The effects of hypoxia and L-lactate on the activity of pre-sympathetic neurons in organotypic brainstem slices

Rat pups (Wistars, 8-10 days old) were used for preparation of organotypic brainstem slices as described previously (3-5). Sensitivity of C1 neurons to hypoxia was assessed using a genetically encoded ratiometric Ca²⁺ sensor TN-XXL (3;6). Catecholaminergic C1 neurons characteristically express transcriptional factor Phox2 and, therefore, can be targeted using viral vectors to express the gene of interest under the control of an artificial promoter PRSx8 – Phox2-activated promoter (7;8). Slices were inspected under a low magnification dissecting microscope and sections (2-3 per animal) cut from the brainstem regions located caudally from the facial nucleus were selected for plating. Apart from C1 group, another notable RVLM population of Phox2-expressing cells constitutes the more rostral group of putative central chemosensitive neurons of the retrotrapezoid nucleus. These are highly sensitive to changes in PCO₂/pH, but do not appear to be intrinsically sensitive to hypoxia (9). In contrast, C1 neurons are highly sensitive to hypoxia and all neurons
tested in the current study displayed robust responses to low $PO_2$ (Fig 2C). Although, this strongly suggests that the neurons we study belong to the preganglionic C1 catecholaminergic RVLM group, in this paper we refer to these cells as ‘putative C1 neurons’. Adenoviral vectors designed to drive the expression of a Ca$^{2+}$ indicator TN-XXL or a reporter protein enhanced green fluorescence protein (EGFP) under the control of PRSx8 promoter were added to the incubation medium at the time of slice culture preparation at $5 \times 10^8$–$5 \times 10^{10}$ transducing units ml$^{-1}$.

Experiments were performed after 7-10 days of incubation. An organotypic slice, containing targeted RVLM neurons targeted to express TN-XXL, was transferred to a recording chamber mounted on an upright Olympus FV1000 microscope (Olympus, Japan), and continuously superfused with HEPES-buffered solution (HBS; containing in mM: NaCl 137, KCl 5.4, Na$_2$HPO$_4$ 0.25, KH$_2$PO$_4$ 0.44, CaCl$_2$ 1.3, MgSO$_4$ 1.0, NaHCO$_3$ 4.2, HEPES 10, Glucose 5.5; pH 7.4; 34±1°C). For excitation, the 456 nm argon laser was used, while emission was detected using 480-520 nm and 535-590 nm filters for CFP and YFP, respectively. [$Ca^{2+}$]$_i$ responses were determined by recording changes in relative fluorescence intensity ($F/F_0$). Hypoxic conditions were induced by gradual replacement of oxygen in the medium with nitrogen or argon. Test drugs were applied 15-30 min before the hypoxic challenge. In a separate series of experiments the effect of L-lactate (2 mM, pH 7.4; Sigma, Poole, UK) on [$Ca^{2+}$]$_i$ in putative C1 neurons was determined as previously described (5). At the end of the recordings, KCl (20 mM) was applied to record the peak [$Ca^{2+}$]$_i$ increases during neuronal depolarization. All data presented were obtained from at least 6 separate preparations. Electrophysiological recordings of putative C1 neurons transduced to express EGFP were performed in current-clamp whole-cell mode at 10
kHz sampling rate using an AxoClamp2B amplifier (Molecular Devices, Sunnyvale, USA), a 1401 interface and Spike 2 software (both from Cambridge Electronic Design Ltd, Cambridge, UK). Recording pipettes (3–5 mΩ) were filled with a solution containing (in mM): potassium gluconate 130, HEPES 10, EGTA 11, NaCl 4, MgCl₂ 2, CaCl₂ 1, ATP 2, GTP 1 and glucose 5.

Measurements of hypoxia-induced release of lactate in the acute brainstem slices

Acute horizontal brainstem slices containing ventral structures of the brainstem (including the RVLM) were prepared as previously described (10). Briefly, male Wistar rats (8-9 weeks of age, 250-320 g body weight, n=10) were terminally anesthetized with halothane inhalation overdose, the brainstem was removed and placed in ice cold artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 26 mM NaHCO₃, 3 mM KCl, 2 mM CaCl₂, 1.25 mM NaH₂PO₄, 1 mM MgSO₄, 10 mM Glucose saturated with 95% O₂ and 5% CO₂ (pH 7.4) with an addition of 9 mM Mg²⁺. Horizontal slice (thickness ~400 µm) containing ventral structures of the brainstem (including the RVLM) was prepared and incubated for 1 h at room temperature in a normal aCSF solution saturated with 95% O₂ and 5% CO₂ (10). Lactate biosensors (2 mm in length and 50 µm in diameter; Sarissa Biomedical, Coventry, UK) were used to record release of lactate from the ventral surface of the medulla oblongata. Recordings were made from the slice placed on an elevated grid in a flow (2 ml min⁻¹) chamber at 37°C. Hypoxic conditions were induced by replacing oxygen in the medium with nitrogen (perfusion of the chamber with aCSF saturated with 95% N₂ and 5% CO₂). Biosensors were calibrated with known amounts of lactate (10 or 100 µM) immediately before and after the recordings. Biosensors measure efflux of
lactate which is reaching the enzyme/polymer layer from the surface of the slice in a perfused chamber with a relatively high flow rate. Although biosensor recordings accurately reflect changes in the release of an analyte of interest (10), in this experimental paradigm the values recorded are likely to underestimate the absolute level of extracellular lactate within the brainstem tissue.

The effect of L-lactate on sympathetic nerve activity and the arterial blood pressure in vivo

Male Wistar rats (8-9 weeks of age, 250-320 g body weight) were anesthetized with pentobarbitone sodium (induction 60 mg kg⁻¹ i.p.; maintenance 10-15 mg kg⁻¹ h⁻¹ i.v.). Core temperature was maintained at 37.0±0.2°C. The trachea was cannulated and the animal was ventilated mechanically (1 Hz, tidal volume 2-2.5 ml) with oxygen-enriched gas mixture (70% N₂/ 30% O₂). The femoral artery and vein were cannulated for monitoring of the arterial blood pressure and administration of drugs, respectively. An adequate level of anesthesia was ensured by maintaining stable levels of the arterial blood pressure, heart rate and the absence of corneal reflexes and flexor responses to a paw-pinch. End-tidal level of CO₂ was monitored continuously (Capstar-100, CWE Inc, USA). Arterial PO₂, PCO₂ and pH were measured regularly and maintained at physiological levels by adjusting the rate of mechanical ventilation and/or tidal volume. The animal was placed in a stereotaxic frame, bilaterally vagotomized and the ventral surface of the medulla oblongata was exposed for application of test compounds as described previously (10;11). To record changes in central sympathetic drive, renal sympathetic nerve was isolated and placed on bipolar silver electrodes (1;2). Renal sympathetic nerve activity
(RSNA) was amplified (x10 K), filtered (800-1000 Hz), rectified and smoothed. After a period of stabilization (~60 min), saline was applied on the exposed brainstem surface followed in ~30 min by application of L-lactate (20 mM, pH 7.4; 30 µl). CSF continuously accumulates on the exposed ventral surface of the brainstem, therefore, all the applied compounds are rapidly diluted. Robust increases in the activity of C1 neurons in vitro were observed after application of 2 mM lactate, therefore, to determine the in vivo effect, lactate was applied in a 20 mM solution to account for rapid dilution by CSF and lactate consumption by the tissue. RSNA mean level of activity was assessed by smoothing the raw signal with a time constant of 10 s. Evoked changes in mean level of activity were normalized with respect to the baseline (100%) and zero level following ganglionic blockade after systemic administration of hexamethonium (20 mg kg⁻¹, i.v.) (Fig 3C).

The effect of facilitated ATP breakdown in the RVLM on the arterial blood pressure in the SHR

To interfere with ATP-mediated signaling by promoting facilitated breakdown of extracellular ATP, a lentiviral (LV) vector was used to drive the expression of a potent membrane-bound ectonucleotidase - transmembrane prostate acidic phosphatase (TMPAP). Efficacy of the transgene in blocking ATP-mediated communication between astrocytes was demonstrated previously (1). Eight-week-old male SHRs (n=16) and age- and sex-matched Wistar rats (n=16) were anesthetized with a mixture of ketamine (60 mg kg⁻¹, i.m.) and medetomidine (250 µg kg⁻¹, i.m.). The animals were placed in a stereotaxic frame and the RVLM was targeted (using the above coordinates) with two microinjections per side (1 µl each, 0.1 µl min⁻¹) of
either EF1α-TMPAP-EGFP-LV (titer $3.8 \times 10^8$ transducing units ml$^{-1}$) or control vector EF1α-EGFP-LV (titer $2.9 \times 10^9$ transducing units ml$^{-1}$). Anesthesia was reversed with atipemazole (1 mg kg$^{-1}$). For post-operative analgesia, rats received daily injections of buprenorphine (0.05 mg kg$^{-1}$ d$^{-1}$; s.c.) for three days and caprofen (4 mg kg$^{-1}$ d$^{-1}$; i.p.) for five days. Arterial blood pressure measurements (tail cuff method, as described above) were performed one week before the injections of viral vectors and were repeated twice weekly for 5 weeks after the viral gene transfer. All measurements were performed by the same investigator between 10:00 and 14:00 hrs. Weekly arterial blood pressure values were obtained by averaging measurements taken on two different days.

*Immunohistochemistry*

At the end of the experiments, rats transduced to express EF1α-TMPAP-EGFP-LV or EF1α-EGFP-LV were terminally anesthetized with urethane (2 g kg$^{-1}$, i.p., and perfused transcardially with saline followed by 500 ml of ice-cold paraformaldehyde (4%). The brains were post-fixed in the same fixative for 24 h, cryoprotected with 30% sucrose for 12 h and sectioned (30 µm) using a freezing microtome. Catecholaminergic RVLM neurons were identified by Tyrosine Hydroxilase (TH)-immunoreactivity (1;2;12). To determine the extent of TMPAP-EGFP expression in the RVLM, the tissue was processed for immunohistochemical detection of GFP. The brainstem slices were incubated in rabbit anti-TH antibody (1:250; Abcam) and chicken anti-GFP antibody (1:250; Aves) for 48 hours followed by incubation in donkey anti-rabbit Alexa 568 and donkey anti-chicken Alexa 488 (1:1000, Molecular
Probes) for 1 hour. Images were taken using a Leica microscope equipped with a high resolution CCD camera.

Data analysis
Recordings of the physiological variables and parenchymal $PO_2$ obtained in anesthetized animals, electrophysiological recordings of RVLM neuronal activity and lactate biosensor measurements were processed using 1401 interface and analyzed using Spike 2 software (Cambridge Electronic Design). Imaging data were collected and analyzed using Olympus software (Olympus). Data are reported as mean ± s.e.m. Datasets were compared by Kruskal–Wallis ANOVA by ranks or Student’s paired or unpaired t test, as appropriate. Differences between groups with p values of <0.05 were considered significant.
Supplemental Reference List


