Differential Role of Kinases in Brain Stem of Hypertensive and Normotensive Rats

Maryam Seyedabadi, Ann K. Goodchild, Paul M. Pilowsky

Abstract—Spontaneously hypertensive rats (SHR) are characterized by extreme elevations of blood pressure. The genetic factors underlying this are yet to be identified. Here we demonstrate, in vivo, that in SHR and normotensive Wistar-Kyoto rats (WKY), injection of the mitogen-activated protein kinase inhibitor PD 098,059 bilaterally into the rostral ventrolateral medulla (RVLM) dramatically lowers arterial pressure, PD 098,059 does not alter the responses evoked by microinjection of glutamate into the RVLM or brief apnea. Wortmannin (phosphatidylinositol-3 kinase inhibitor) bilaterally into the RVLM causes a 35±4% fall in arterial pressure in SHR but has no effect in WKY. Furthermore, wortmannin reduces the pressor response evoked by microinjection of angiotensin (Ang) II in the RVLM of SHR compared with WKY. The response to Ang II microinjection into the RVLM of WKY was unaffected by wortmannin. Simultaneous bilateral injections of PD 098,059 and wortmannin into the RVLM abolished the response to exogenous Ang II in the RVLM but did not affect the response evoked by glutamate in either SHR or WKY. Thus, it appears that PD 098,059– and/or wortmannin-sensitive mechanisms are not involved in the responses evoked by glutamate in the RVLM and that these kinase inhibitors are not neurotoxic. We conclude that a PD 098,059–sensitive pathway in the RVLM of SHR and WKY tonically regulates arterial pressure and that a wortmannin-sensitive pathway in the RVLM is important in the maintenance of hypertension in SHR. This may be related to a phosphatidylinositol-3 kinase–dependent mechanism involved in the action of Ang II on the Ang II type 1 receptor. (Hypertension. 2001;38:1087-1092.)

Key Words: rats, inbred SHR □ arterial pressure □ protein kinases □ angiotensin II □ brain

Spontaneously hypertensive rats (SHR) develop hypertension,1 obesity,2 and impaired glucose tolerance.3 These features mimic those identified as syndrome X in humans. Even though obesity and impaired glucose tolerance have recently been linked to a defect in the enzyme fatty acid translocase,4 the cause of the hypertension remains elusive, although some previous work suggests increases in efferent sympathetic nerve activity in SHR.5,6

Recent studies on cell cultures derived from the hypothalamus/brain stem of neonatal Wistar-Kyoto rats (WKY) and SHR7 report that mitogen-activated protein (MAP) kinase activity is essential for the angiotensin (Ang) II–induced transcription of catecholamine-synthesizing enzymes, such as tyrosine hydroxylase and dopamine-β-hydroxylase, and the norepinephrine transporter. Yang and Raizada8 further showed that in cultures from SHR, there is a MAP kinase–independent, phosphatidylinositol 3 (PI3) kinase–dependent, Ang II–signaling pathway that is not operative in cultures from control rats (WKY). Although these studies suggest a biochemical difference between the 2 rat strains, there is no evidence to date that this difference has any physiological significance or indeed whether it plays a role in the increase in arterial pressure seen in SHR. In the present study, we provide evidence that a PD 098,059 (inhibitor of MAP kinase)–sensitive pathway in the rostral ventrolateral medulla (RVLM) of the brain stem is vital for maintaining tonic levels of arterial pressure and for the expression of some, but not all, drug-induced changes in arterial pressure regulation. Furthermore, we show that a wortmannin (inhibitor of PI3 kinase)-sensitive pathway in the RVLM may be a cause of the elevated arterial pressure in SHR.

Methods

All procedures in the present study were in accordance with the requirements of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the local animal care and ethics committee. Male adult SHR and WKY, age 18 to 20 weeks, weighing 350 to 430 g, were used.

Rats were anesthetized with urethane (1.3 g/kg IP). The right femoral vein and artery were cannulated for drug and fluid administration (pancuronium [2 mg/mL] and 1 mL of saline containing 5% glucose/h) and arterial pressure recording, respectively. The trachea was cannulated, and the rats were mechanically ventilated. Rats were placed in a stereotaxic frame with the head flexed at ~30°, and the dorsal surface of the medulla was exposed. The animals were paralyzed after a pressor site was identified in the RVLM by using...
glutamate microinjections (50 nL, 100 mmol/L in 10 mmol/L PBS, pH 7.4). The level of anesthesia was checked by observing only small changes in arterial pressure in response to a firm paw pinch at intervals throughout the experiment. Administrations of additional doses of urethane (5% to 10% of original) were made if required.

Drug Microinjections
Pressure microinjections of drugs into the brain stem were made from multibarrel glass electrodes (PMP-100 multibarrel puller, Micro Data Instruments). All injections were of 50 nL so as to provide an effective compromise between excessive spread of the drug and coverage of the area being examined.

Drugs injected were PD 098,059 (50 μmol/L, Sigma-RBI), the selective noncompetitive antagonist of the MAP kinase activator, MAP kinase kinase, and wortmannin (100 mmol/mL, Sigma-Aldrich), a potent, selective, irreversible PI3 kinase inhibitor. The vehicle used to dissolve both PD 098,059 and wortmannin was 1% ethanol in PBS (10 mmol/L phosphate buffer and 0.9% NaCl, pH 8.0). Drug concentrations were chosen in accordance with those of previous in vitro studies.

Ang II (4 mmol/L, Auspep) was dissolved in PBS (10 mmol/L, pH 7.4, 3 parts), and BSA adsorbed to colloidal gold (Sigma, 1 part). The target sites for further drug injection were those at which glutamate evoked responses >40 mm Hg. The coordinates used for targeting the RVLM in the present study were 0.6±0.2 mm rostral to the obex, 2±0.2 mm lateral to the midline, and 3.8±0.5 mm ventral from the surface. After injection on one side, the pipette was withdrawn and moved to the contralateral side. Injections were made within 1 to 2 minutes of each other.

Experimental Design
Arterial pressure was initially monitored for 14 hours after drug injections to determine whether any effects due to transcriptional and translational events occurred.

Periods of apnea were used to determine the ability of the neurons within the RVLM to respond to a naturalistic physiological stimulus. Apnea was evoked regularly by turning the ventilator off for 5 seconds to elicit a brief hypercapnic hypoxia. Ang II and glutamate were injected into the RVLM at the end of each experiment to test the effect of the drugs or vehicle on the pressor responses evoked. On completion of the experiment, the brain stem was removed, fixed (in 4% formaldehyde in 0.1 mol/L phosphate buffer and 0.9% NaCl, pH 7.4), and 7.4, 3 parts), and BSA adsorbed to colloidal gold (Sigma, 1 part).

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Statistical Analysis
Arterial pressure was monitored continuously, and the mean arterial pressure at set intervals (averaged over 5 minutes) was obtained offline. Grouped data are expressed as mean±SE arterial pressure and/or percentage change in mean±SE arterial pressure. The effect of drug treatments on the arterial pressure of SHR was compared with WKY, and the effect of each drug treatment on arterial pressure was compared with the effect of vehicle treatment in each strain by unpaired 2-tailed Student’s t tests. A paired 2-tailed Student’s t test was used to investigate the effect of the drug on that animal/group. A value of P<0.05 was considered statistically significant.

Results
Brief Apnea
Figure 1, panels A2 to A4, shows the effect on arterial pressure evoked by apnea. Turning the ventilator off caused a small transient increase in arterial pressure that was similar before and after drug treatment (Figure 1, panel A2). Grouped data are shown in Figure 1, panels A3 (WKY) and A4 (SHR). The pressor response evoked by apnea was not significantly different in WKY and SHR. The pressor responses to apnea in both WKY and SHR were never affected by the drug treatments.

Drug Treatments
Vehicle Microinjections
The effect of vehicle (1% ethanol in 10 mmol/L PBS) microinjected bilaterally into the RVLM was examined in SHR (n=4) and WKY (n=4). Before microinjections, the mean arterial pressure of SHR was 157±7 mm Hg and that of WKY was 101±3 mm Hg (P=0.0001). Vehicle injections had no effect on arterial pressure in either WKY or SHR, except occasionally, when a very small transient pressor response was evoked immediately on injection. The mean arterial pressure 14 hours after vehicle injection was 153±6 mm Hg in SHR and 105±4 mm Hg in WKY.

PD 098,059
Bilateral PD 098,059 injections into the RVLM caused a gradual fall in the arterial pressure in both WKY and SHR (Figure 2, panels A1 to A3). Mean arterial pressure fell from 111±4 to 71±10 mm Hg in WKY (n=8, P=0.0021) and from 157±7 to 100±6 mm Hg in SHR (n=4, P<0.0001). Figure 2, panel A3, illustrates the percentage fall in mean arterial pressure at 0.25, 3, and 7 hours after the injections. By 7 hours, PD 098,059 caused a similar percentage fall in mean arterial pressure in both strains.
arterial pressure in both SHR and WKY (36±4% in SHR and 34±10% in WKY).

**Wortmannin**
Bilateral injections of wortmannin into the RVLM of WKY had no effect on mean arterial pressure (from 100±5 to 101±5 mm Hg, n=4; Figure 2, panels B1 and B3) over the 7-hour period. In contrast, in SHR, bilateral wortmannin injection into the RVLM caused a gradual fall in mean arterial pressure from 149±8 to 99±1 mm Hg (35±4%, n=4, P<0.0001; Figure 2, panel B3) over 7 hours.

**PD 098,059 and Wortmannin**
Bilateral PD 098,059 injections caused a similar decrease in arterial pressure in both SHR (n=4) and WKY (n=4) up to 7 hours after drug treatment (Figure 2, panels A1 to A3), whereupon both PD 098,059 and wortmannin were injected together bilaterally. This caused a further drop in mean arterial pressure in SHR but had no effect in WKY (Figure 2, panel C). Seven hours after PD 098,059 and wortmannin, mean arterial pressure was similar in both strains (62±2 mm Hg in SHR and 65±9 mm Hg in WKY).

**Glutamate**
Responses to unilateral glutamate microinjection in the RVLM were determined before and after drug treatment in all animals. The pressor response evoked by glutamate microinjection in the RVLM was similar in both WKY (52±3 mm Hg, n=16) and SHR (55±2 mm Hg, n=12) (Figure 3, panel A). Furthermore, the response to glutamate was not affected by vehicle, PD 098,059, and/or wortmannin in either WKY or SHR (Figure 3, panel A). Therefore, although the animals treated with PD 098,059 and wortmannin had a lower arterial pressure, the pressor response to glutamate microinjections made into the RVLM were not altered in magnitude compared with those evoked before drug treatment.

**Ang II Microinjections**
Unilateral microinjection of Ang II into the RVLM of WKY (Figure 3, panels B1 and B3) and SHR (Figure 3, panels B2 and B3) elicited pressor responses 14 hours after the microinjection of vehicle into the RVLM in both. Furthermore, the pressor response evoked by Ang II after vehicle in SHR (26±3 mm Hg, n=3) was significantly greater than that evoked in WKY (13±1 mm Hg, n=3, P<0.01; Figure 3, panel B3, vehicle). Although the glutamate response was unaffected by injection of the kinase inhibitors into the RVLM (Figure 3, panel A), the response to Ang II was markedly attenuated by wortmannin in SHR (from 26±3 to 9±1 mm Hg, n=3, P=0.0041) but was unaffected in the WKY (n=4) (Figure 3, panel B3, wortmannin). The Ang II evoked pressor response after wortmannin injection in SHR was comparable to the response evoked by Ang II in the RVLM in WKY with or without wortmannin injection. In contrast, the Ang II response was completely abolished in both SHR (n=3) and WKY (n=3) after simultaneous injection of both PD 098,059 and wortmannin (Figure 3, panel B3, PD 098,059+wortmannin). The effect of PD 098,059 on the Ang II pressor response alone was not tested.

Figure 2. Effect of PD 098,059, wortmannin, and both drugs bilaterally injected into the RVLM of WKY and SHR. Carets indicate times of injections. A1 and A2, AP traces from a WKY and an SHR, respectively, in which PD 098,059 was injected every 4 hours. A3, Grouped data showing that a significant and similar fall in MAP in both SHR (n=4) and WKY (n=8) was evoked. B1 and B2, AP traces from WKY and SHR, respectively, in which wortmannin was injected every 4 hours. B3, Grouped data showing that mean AP (MAP) in SHR was 34±5% lower 7 hours after the first wortmannin microinjection, with no change evoked in WKY. C, In SHR (n=4) and WKY (n=4), microinjections of PD 098,059 were made every 4 hours for 14 hours. Wortmannin was also microinjected bilaterally every 4 hours from t=8 hours. Seven hours after the initial PD 098,059 microinjections, there was a significant fall in MAP in both SHR (32±2%) and WKY (29±2%). On simultaneous injections of PD 098,059 and wortmannin (from t=8 hours to t=14 hours), there was a further drop at t=14 hours (33±2%) in MAP in only the SHR. *Significantly different from preceding time point.
Our results with PD 098,059 and wortmannin must be treated cautiously, because kinase inhibitors have rarely been used in vivo\textsuperscript{13,14}; thus, their effects have not been evaluated. The present study shows for the first time that localized use of specific kinase inhibitors, at the concentrations used in the present study, do not appear to exhibit cytotoxic effects. Kinase inhibitors injected into the RVLM affected neither the response to apnea nor the pressor response evoked by glutamate injected into the RVLM, at least within 14 hours of injection under the prevailing anesthetic conditions. Furthermore, histological examination revealed an apparently normal cellular appearance.

In vitro studies by Yang and colleagues\textsuperscript{7,8,11,15} have shown that one role, played by MAP kinase and PI3 kinase, was regulation of the transcription and translation of catecholaminergic biosynthetic enzymes and transporters. The duration of the experiments in the present study (up to 14 hours) allowed time for any possible transcriptional and translational consequences to take place after kinase inhibition.\textsuperscript{16} Thus, the effects seen in the present study may be a result of changes in the expression of catecholamine-related, or other, gene products. Vehicle injection showed that the duration of the experiments did not affect the viability of the preparation and that arterial pressure can be maintained in a homeostatic state over at least 14 hours in this preparation.

Inasmuch as the vehicle did not affect arterial pressure, the falls in arterial pressure seen after the injection of PD 098,059 in both WKY and SHR and wortmannin in SHR are most likely to be due to the inhibition of the kinases, for which the drugs are thought to be effective and selective at the doses used. Furthermore, the lack of effect of wortmannin in WKY shows that the effects were not due to some global or nonspecific inhibition. The roles of nonneuronal elements within the RVLM in the present study require further investigation.

The fall in arterial pressure evoked by PD 098,059 was of a similar magnitude in both WKY and SHR, indicating that the PD 098,059–sensitive mechanism in both strains is of equal importance. This is consistent with an in vitro study that has reported first that MAP kinase activity is similar in neuronal cultures derived from the brain stem and hypothalamus of both SHR and WKY.\textsuperscript{8,11} Furthermore, the ratio of the activated GTP-Ras to the inactive GDP-Ras (a protein upstream from MAP kinase) was similar in both strains of rats.\textsuperscript{11}

In contrast to the hypotensive effect of PD 098,059 in both SHR and WKY, wortmannin was effective only in the SHR. The hypotensive effect of wortmannin in the SHR was comparable to that evoked by PD 098,059 in either SHR or WKY.

Results from experiments with both PD 098,059 and wortmannin showed that the fall in arterial pressure in the SHR was significantly larger than the fall evoked by PD 098,059 alone. Taken together, these findings suggest that PI3 kinase is not active in the RVLM in WKY. That the response to Ang II in the RVLM is not affected by wortmannin in the WKY supports this suggestion, as do the results of Yang and colleagues\textsuperscript{7,15} who demonstrated that the responses evoked by Ang II stimulation of cell cultures from WKY could be completely blocked by MAP kinase inhibition.

Discussion

In the present study, we show, for the first time, that there is a tonically active PD 098,059–sensitive pathway present in the RVLM in both SHR and WKY that plays a vital role in the maintenance of arterial pressure. Furthermore, the findings in the present study demonstrate for the first time that microinjections of wortmannin into the RVLM cause a fall in arterial pressure in SHR but not WKY. Thus, there appears to be no wortmannin-sensitive pathway in WKY at least in this preparation. To our knowledge, this is the first time that these pathways have been shown, in vivo, to play a physiological role in the central regulation of arterial pressure.
Interestingly, the arterial pressure attained after wortmannin in the RVLM of SHR was not different from that of untreated or vehicle-treated WKY. This effect suggests one possible mechanism for the hypertension that is characteristic of the SHR. The RVLM is 1 of 5 main groups of presympathetic neurons from which sympathetic output originates. Significant increases in the discharge rate and differences in electrophysiological properties of RVLM neurons in SHR compared with WKY have been reported, indicating that this region may play a role in the genesis of the hypertension seen in the SHR.

The magnitude of the pressor response evoked by brief apnea and by glutamate activation in the RVLM was similar in WKY and SHR. This is consistent with some previous studies; however, others have reported that the pressor response and increase in sympathetic activity elicited by glutamate or N-methyl-D-aspartate injections into the RVLM in the SHR were significantly greater than those in the WKY. In contrast to glutamate, the pressor response evoked by Ang II in the RVLM was considerably smaller in WKY than in SHR in the present study. It has been reported that the response to Ang II in the RVLM is not significantly different between the 2 strains. However, there are studies that support the present finding. First, Chan et al reported an enhanced sensitivity and responsiveness of single neurons in the RVLM to Ang II in SHR. Second, there are more Ang II type 1 receptors in the brain stem/hypothalamus of SHR than of WKY. Similarly, in neuronal cultures taken from the brain stem and hypothalamus, noradrenaline uptake and tyrosine hydroxylase activity after Ang II stimulation were significantly greater in SHR than in WKY.

In the present study, although the kinase inhibitors used did not alter the response to glutamate, they did influence the pressor response evoked by Ang II microinjections in the RVLM. In SHR, wortmannin reduced the Ang II response to levels seen in WKY. Combined injections of PD 98059 and wortmannin into the RVLM abolished the response to Ang II in both SHR and WKY. The simplest explanation for these findings is that in SHR both a PI3 kinase–dependent pathway and a MAP kinase–dependent pathway in the RVLM are necessary to evoke an Ang II–induced response but that in WKY, only a MAP kinase–dependent pathway is needed. These physiological findings complement the biochemical studies of Yang and Raizada, who found that Ang II stimulation of neuronal cultures resulted in an increase in production of the catecholamine-synthesizing enzymes tyrosine hydroxylase and dopamine-β-hydroxylase and the norepinephrine transporter. This Ang II–induced effect was abolished by MAP kinase inhibition in WKY but required both PI3 kinase and MAP kinase inhibition for complete blockade in cultures from SHR. Whether the effects seen in the present study are due to the modification of intracellular signaling pathways for transcriptional and translational pathways of catecholamine-synthesizing enzymes and transporters, as suggested by the work of Yang and Raizada, remains to be clarified.

These findings suggest that inhibition of PI3 kinase may be a new therapeutic target in the treatment of hypertension. It is especially interesting that inhibition of PI3 kinase reduced arterial pressure to the levels seen in normotensive animals but did not cause attenuation of normal homeostatic reflexes. Equally important in this context is the lack of effect of PI3 kinase inhibition in WKY.

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References


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