Up-regulation of vascular and renal mitogen-activated protein kinases in hypertensive rats is normalized by inhibitors of the Na\(^+\)/Mg\(^{2+}\) exchanger

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ABSTRACT

In the present in vivo study, we have investigated whether inhibitors of the Na\(^+\)/Mg\(^{2+}\) exchanger quinidine and imipramine influence the development of hypertension and whether this is associated with modulation of mitogen-activated protein (MAP) kinase activation in arteries and kidneys of hypertensive rats. Sprague–Dawley rats were divided into four groups (n = 6/group): control (vehicle), angiotensin II (Ang II; 150 ng/kg of body weight per min subcutaneously), quinidine [Ang II (150 ng/kg of body weight per min) + quinidine (5 mg/kg of body weight per day in food)] and imipramine groups [Ang II (150 ng/kg of body weight per min) + imipramine (5 mg/kg/day in food)]. Rats were studied for 3 weeks. Phosphorylation of vascular and renal extracellular-signal-regulated protein kinase 1/2 (ERK1/2), p38MAP kinase and c-Jun N-terminal kinase (JNK) were assessed using phospho-specific antibodies. Ang II increased systolic blood pressure from 112 ± 5 mmHg to 215 ± 9 mmHg (P < 0.01). Development of hypertension was attenuated in Ang II-infused rats treated with quinidine (173 ± 6 mmHg) and imipramine (152 ± 6 mmHg) (P < 0.01). Phosphorylation of ERK1/2, p38MAP kinase and JNK, which were increased 2–3-fold in arteries of the Ang II group, were reduced by quinidine and imipramine (P < 0.05). Activation of renal MAP kinases was also increased in the Ang II group (P < 0.05). Quinidine and imipramine reduced the phosphorylation of renal ERK1/2, but did not modify renal p38MAP kinase or JNK. Our data demonstrate that Ang II induces severe hypertension in Sprague–Dawley rats and this is associated with increased phosphorylation of vascular and renal MAP kinases. Quinidine and imipramine attenuated the development of hypertension and normalized MAP kinase activity. The findings from this study suggest a possible role for the Na\(^+\)/Mg\(^{2+}\) exchanger in vascular signalling events associated with blood pressure elevation in Ang II-dependent hypertension.

INTRODUCTION

A major haemodynamic factor contributing to the pathogenesis of hypertension is increased vascular resistance due mainly to functional and structural changes of peripheral resistance arteries [1]. Functional alterations include enhanced vascular reactivity and/or impaired vasodilation [1, 2]. Structural changes include reduced lumen diameter and vascular wall thickening (vascular remodelling) and involve vascular smooth muscle cell

Key words: blood pressure, imipramine, kidney, Mg\(^{2+}\), quinidine, protein kinases, resistance arteries.

Abbreviations: Ang II, angiotensin II; [Ca\(^{2+}\)]\(_i\), intracellular free Ca\(^{2+}\) concentration; ERK1/2, extracellular-signal-regulated kinase 1/2; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; [Mg\(^{2+}\)]\(_i\), intracellular free Mg\(^{2+}\) concentration; [Na\(^+\)]\(_i\), intracellular free Na\(^+\) concentration; SBP, systolic blood pressure; VSMC, vascular smooth muscle cell.

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(VSMC) growth and increased deposition of extracellular matrix components [3,4]. Renal dysfunction, due to nephrosclerosis, interstitial fibrosis and inflammation, is also associated with development of hypertension [1,5]. Many of these processes are influenced by Mg$^{2+}$.

In vitro and in vivo studies have demonstrated that increased concentrations of extracellular Mg$^{2+}$ cause vasodilation and attenuate agonist-induced vasoconstriction, whereas reduced concentrations of Mg$^{2+}$ have the opposite effects, causing enhanced contraction and impaired vasorelaxation [6,7]. Mg$^{2+}$ probably influences these events by regulating intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}]_i$), a key determinant of vascular smooth muscle contraction. Mg$^{2+}$ modulates Ca$^{2+}$ entry, mobilization, binding and translocation in VSMCs [8,9]. Reduction from sarcoplasmic reticular stores and regulates Ca$^{2+}$ events by regulating intracellular free Ca$^{2+}$.

Recent studies using microarray technology have demonstrated that changes in cellular inflammation [14]. Increase in extracellular Mg$^{2+}$ activates [Ca$^{2+}]_i$, whereas elevation in Mg$^{2+}$ lowers [Ca$^{2+}]_i$ [8,10]. Mg$^{2+}$ displaces and competes with Ca$^{2+}$ at cell membranes for functional binding sites, inhibits Ins(1,4,5)$P_3$-induced Ca$^{2+}$ mobilization from sarcoplasmic reticular stores and regulates Ca$^{2+}$ sequestration and efflux through Mg$^{2+}$-dependent activation of Ca$^{2+}$-ATPase [8,11–13]. Collectively, these data indicate that Mg$^{2+}$ has numerous Ca$^{2+}$-antagonistic properties, which could contribute to altered [Ca$^{2+}]_i$, and increased vessel reactivity and tone in hypertension.

In addition to regulating vascular smooth muscle contraction and dilation, Mg$^{2+}$ influences cellular processes associated with vascular remodelling and renal fibrosis, characteristic features in hypertension. At the subcellular level, these effects may occur, at least in part, through Mg$^{2+}$-dependent regulation of mitogen-activated protein (MAP) kinases, important signalling molecules involved in cell differentiation, cell proliferation, cell death and inflammation [14]. Recent studies using microarray technology have demonstrated that changes in cellular Mg$^{2+}$ have potent modulatory effects on expression of various signalling kinases, including the MAP kinases [15,16]. To date at least six mammalian MAP kinase subfamilies have been identified, of which extracellular signal-regulated protein kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38MAP kinase are the best characterized [14]. ERK1/2 is a major growth signalling kinase, whereas JNK and p38MAP kinase influence cell survival, apoptosis, differentiation and inflammation [17,18]. Increased activation of MAP kinases has been demonstrated in cardiac, vascular and renal tissue of hypertensive rats and appears to be important in cardiovascular remodelling, renal fibrosis and inflammation in hypertension [19–22]. Because Mg$^{2+}$ modulates activity of many enzymes, including MAP kinases, it is possible that disturbances in Mg$^{2+}$ metabolism in hypertension could contribute to altered regulation of MAP kinases and consequently to vascular and renal functional and structural changes.

Findings from experimental and clinical studies demonstrate, for the most part, a decrease in plasma and tissue Mg$^{2+}$ levels in hypertensive rats and patients compared with normotensive control counterparts [8,23–25]. Although mechanisms underlying alterations in cellular Mg$^{2+}$ status in hypertension are unclear, changes in transmembrane Mg$^{2+}$ transport could play a role. Previous studies [26,27] have demonstrated hyperactivation of Na$^+$/Mg$^{2+}$ exchange activity in hypertension, where Mg$^{2+}$ efflux is coupled with Na$^+$ influx. We have reported [28] that activity of Na$^+$-dependent Mg$^{2+}$ transport is increased in VSMCs from spontaneously hypertensive rats. In addition, we have reported recently [29] that the inhibition of the Na$^+$/Mg$^{2+}$ exchanger attenuates blood pressure elevation in hypertensive rats.

In the present in vivo study, we sought to determine whether altered regulation of vascular and renal MAP kinases in hypertension is associated with processes linked to Na$^+$-dependent Mg$^{2+}$ exchange. To this end, we have investigated the effects of quinidine and imipramine, prototypical inhibitors of Na$^+$/Mg$^{2+}$ exchanger activity, on the phosphorylation of ERK1/2, p38MAP kinase and JNK in renal cortex and resistance arteries of hypertensive rats. We and others have shown previously [28–30] that quinidine and imipramine potently inhibit Na$^+$-dependent Mg$^{2+}$ transport in VSMCs, renal-derived cells and platelets. The present study was performed in angiotensin II (Ang II)-induced hypertensive rats. This model was selected because: (i) hypertension develops rapidly [29]; (ii) this model is Mg$^{2+}$-sensitive [29]; and (iii) Ang II is known to modulate the Na$^+$/Mg$^{2+}$ exchanger in vascular and renal cells [28,30–33].

**MATERIALS AND METHODS**

**Experimental animals**

The study was approved by the Animal Ethics Committee of the Clinical Research Institute of Montreal (IRCM) and carried out according to the recommendations of the Canadian Council for Animal Care. Adult male Sprague–Dawley rats (16-weeks old, n = 24) were studied. They were randomly divided into four groups: (i) control (receiving vehicle by mini-osmotic pump; Alzet Osmotic Pumps, Cupertino, CA, U.S.A.), (ii) Ang II (150 ng/kg of body weight per min administered by mini-pump), (iii) quinidine [Ang II (150 ng/kg of body weight per min) and quinidine (5 mg/kg of body weight per day in drinking water)] and (iv) imipramine [Ang II (150 ng/kg of body weight per min) and imipramine (5 mg/kg of body weight per day in drinking water)]. Concentrations of quinidine and imipramine were selected according to published data [34,35]. Rats were treated for 3 weeks. They were maintained on standard rat chow and given drinking water ad libitum. Systolic blood pressure (SBP) was measured weekly by the tail-cuff method. Tail venous blood was withdrawn at 3 weeks for determination of plasma concentrations of Mg$^{2+}$, Ca$^{2+}$, K$^+$ and Na$^+$. 

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**Determination of plasma Mg\(^{2+}\), Ca\(^{2+}\), K\(^{+}\) and Na\(^{+}\) levels**

Heparinized blood was centrifuged and the plasma removed for analysis. Plasma levels of Mg\(^{2+}\) and Ca\(^{2+}\) were determined by atomic absorption spectroscopy, and plasma concentrations of K\(^{+}\) and Na\(^{+}\) were determined by flame photometry at the hospital laboratory.

**Western blotting of MAP kinases**

Segments of small mesenteric arteries, which are resistance arteries that contribute to blood pressure regulation, and the cortex of each kidney were homogenized and proteins were prepared for immunoblots as described previously [36]. After denaturation at 100 °C for 5 min, proteins (10 µg for ERK1/2 and 25 µg for p38MAP kinase and JNK) were loaded on to an SDS/12% (w/v) polyacrylamide gel and transferred on to a PVDF membrane (Boehinger Mannheim, Laval, Quebec, Canada) for 1 h at 100 V and 4 °C. Membranes were blocked in milk washing solution {5% (w/v) non-fat dried milk in Tris-buffered saline [19.8 mM Tris/HCl (pH 7.5) and 41 mM NaCl] containing 0.1 % Tween 20} and incubated for 24 h at 4 °C with phospho-specific antibodies to ERK1/2 (1:1000; Calbiochem, La Jolla, CA, U.S.A.), p38MAP kinase (1:1000; Calbiochem) and JNK (1:1000; Calbiochem). To determine total protein content of the MAP kinases, membranes were also probed with antibodies (1:1000) to ERK1/2, JNK and p38MAP kinase. Membranes were washed, incubated with goat anti-(rabbit IgG)–horseradish peroxidase conjugated antibodies (Bio-Rad Laboratories, Hercules, CA, U.S.A.) diluted 1:5000 for 1 h and washed extensively. Membranes were incubated with Blotting Substrate (Boehinger Mannheim), exposed to film and developed. Band intensity was analysed using Image Quant software 5.0 (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

**Statistical analysis**

Results are presented as means ± S.E.M. and compared by ANOVA or by Student’s t test, where appropriate. Tukey–Kramer’s correction was used to compensate for multiple testing. \(P < 0.05\) was significant.

**RESULTS**

**Body weight and blood pressure**

Body weight increased progressively in all groups with no significant differences after 3 weeks of treatment between control (382 ± 12 g), Ang II (383 ± 8 g), quinidine (390 ± 4 g) and imipramine (368 ± 6 g) groups. These findings demonstrate that all rats were in good health and that they thrived for the duration of the study.

**Plasma cation levels**

As shown in Figure 2, plasma levels of Mg\(^{2+}\) and K\(^{+}\) were significantly reduced, whereas plasma Na\(^{+}\) was significantly increased in Ang II-infused rats. Although there was a trend for plasma Ca\(^{2+}\) to increase in the Ang II group, significance was not achieved. In Ang II-infused rats treated with quinidine or imipramine, plasma Mg\(^{2+}\) and K\(^{+}\) concentrations were significantly higher and plasma Na\(^{+}\) was significantly lower compared with untreated Ang II-infused rats. Plasma cation levels in quinidine and imipramine groups were similar to levels in controls.

**Phosphorylation of vascular and renal MAP kinases**

Findings from immunoblotting studies demonstrated that phosphorylation (an index of activation) of ERK1/2,
Figure 2  Effects of Ang II, quinidine and imipramine on plasma cation levels at 3 weeks
Data are presented as the means ± S.E.M., n = 6 rats/group. *P < 0.05 compared with the control, quinidine and imipramine groups.

p38MAP kinase and JNK was significantly increased (P < 0.01) in mesenteric arteries from rats in the Ang II group compared with controls (Figure 3). Treatment of Ang II-infused rats with quinidine or imipramine was associated with significantly reduced phosphorylation of vascular ERK1/2, p38MAP kinase and JNK compared with the Ang II group (Figure 3). There was no difference in MAP kinase expression between groups as detected by Western-blot analysis with anti-total ERK1/2, p38MAP kinase and JNK antibodies (Figure 3, left-hand panels). Protein loading between samples was identical, as assessed by Ponceau S Red staining of membranes.

Phosphorylation of renal MAP kinases was increased 2–3-fold in Ang II rats compared with control counterparts (Figure 4). In quinidine and imipramine groups, the magnitude of renal ERK1/2 activation was significantly reduced compared with the Ang II group. Phosphorylation of renal p38MAP kinase and JNK was increased in the quinidine and imipramine groups compared with controls and was not significantly different compared with that in the Ang II group. There was no difference in expression of total ERK1/2, p38MAP kinase and JNK between groups (Figure 4, left-hand panels).

DISCUSSION
The major findings from our present study demonstrate that augmented phosphorylation of ERK1/2, p38MAP kinase and JNK in peripheral resistance arteries and kidneys of Ang II-induced hypertensive rats are reduced, to varying degrees, by quinidine and imipramine treatment. These effects were associated with normalization of serum Mg^2+, K^+ and Na^+ levels and attenuation in the development of hypertension. Collectively, these data suggest a possible role for quinidine- and imipramine-sensitive Na^+-dependent Mg^2+ exchange activity in the pathophysiology of hypertension. Mechanisms underlying this phenomenon may be related, to some extent, to the modulation of renal and vascular MAP kinase-dependent signalling pathways, important processes associated with renal damage and vascular remodelling in hypertension.

Ang II induced a rapid increase in blood pressure, which was sustained at levels above 200 mmHg after 3 weeks of infusion. This was associated with hypomagnesaemia, hypokalaemia and hypernatraemia. Processes contributing to these cation changes may relate to hyperaldosteronism effects secondary to increased Ang II. Aldosterone stimulates renal Mg^2+ and K^+
excretion and promotes Na\(^+\) reabsorption [37,38]. Accordingly, activation of the Ang II–aldosterone axis leads to increased renal loss of Mg\(^{2+}\) and K\(^+\) and decreased Na\(^+\) excretion with resultant hypomagnesaemia, hypokalaemia and hypernatraemia, as shown in the present study. These cation changes may also be due to direct actions of Ang II, which promotes Mg\(^{2+}\) efflux and Na\(^+\) influx by stimulating Na\(^+\)/Mg\(^{2+}\) exchanger activity. In rats treated with quinidine and imipramine, development of hypertension was attenuated and plasma cation levels were normalized, supporting a role for Na\(^+\)-dependent Mg\(^{2+}\) transport in these processes.

Altered regulation of cellular Mg\(^{2+}\) and Na\(^+\) may have several effects on vascular function and structure in hypertension. In isolated arteries and cultured VSMCs from hypertensive rats, decreased [Mg\(^{2+}\)] may be associated with increased contractility and endothelial dysfunction, probably through modulation of Ca\(^{2+}\)-dependent signalling pathways and induction of oxidative stress [6,8,10]. Recent studies [39–41] suggest that changes in [Mg\(^{2+}\)] and [Na\(^+\)], also influence signalling pathways associated with cell growth, apoptosis and inflammation, notably the MAP kinases. In the present study vascular and renal ERK1/2 phosphorylation was significantly augmented in Ang II-induced hypertensive rats. Hyperactivation of ERK1/2 has been implicated in cellular growth processes leading to vascular and renal hypertrophy in hypertension [19–22]. Increased ERK1/2
phosphorylation also plays a role in enhanced vascular contractility in hypertension [36]. In the quinidine- and imipramine-treated rats, vascular and renal ERK1/2 phosphorylation was significantly reduced compared with untreated Ang II-infused rats. These findings suggest that inhibition of Na\(^+\)/Mg\(^{2+}\) exchange activity protects against up-regulation of ERK1/2-dependent signalling events in renal and vascular cells, which could contribute to vascular regression and improved renal function in quinidine- and imipramine-treated hypertensive rats, as we reported recently [29]. Mechanisms whereby quinidine and imipramine influence MAP kinase activity probably occur through modulation of intracellular cation status via the inhibition of Na\(^+\)/Mg\(^{2+}\) exchanger activity. Although there is evidence that changes in [Na\(^+\)] and [Mg\(^{2+}\)] are associated with altered MAP kinase activity [15,16,39], the exact molecular mechanisms underlying these processes await clarification.

Activation of the other major MAP kinases, p38MAP kinase and JNK, has also been shown to be augmented in hypertension [20,42]. Our findings in the present study are in agreement with previous studies. Increased phosphorylation of p38MAP kinase and JNK is associated with cardiac hypertrophy, vascular inflammatory responses and renal fibrosis in hypertension [17,20,36,42]. Exact mechanisms underlying these processes remain unclear, but oxidative stress (increased

Figure 4  Effects of quinidine and imipramine on MAP kinase activation in kidneys of Ang II-infused rats
After 3 weeks of treatment, protein were extracted from segments of renal cortices and prepared for immunoblotting using anti-(phospho-specific MAP kinase) antibodies to assess MAP kinase activation and anti-(total MAP kinase) antibodies to determine MAP kinase expression. Left-hand panels, representative immunoblots demonstrating phosphorylated fractions and total content of ERK1/2, p38MAP kinase (p38MAPK) and JNK in extracts from kidneys of single rats from control (Cont), Ang II, quinidine (Quin) and imipramine (Imip) groups. Right-hand panels, cumulative data from six experiments. Data are expressed as the percentage protein phosphorylation relative to control conditions, taken as 100 %, and are means ± S.E.M. *P < 0.05 compared with the control group; +P < 0.05 compared with the Ang II group.
bioavailability of oxygen-derived free radicals) might play a role, since p38MAP kinase and JNK are redox-sensitive MAP kinases [43,44] and oxidative stress is increased in hypertension [45]. In addition, we demonstrate in the present study that Na⁺/Mg²⁺ exchanger activity may be important, since quinidine and imipramine normalized vascular p38MAP kinase and JNK activation. In fact, decreased [Mg²⁺], secondary to activation of Na⁺-dependent Mg²⁺ transport, is associated with increased oxidative stress [46], which may, in turn, influence the phosphorylation of p38MAP kinase and JNK.

Although it is possible that improvement in blood pressure itself could contribute to normalization of MAP kinase activity, this is probably not the case in the present study, since phosphorylation of renal JNK and p38MAP kinase were not significantly altered in the present study, since phosphorylation of renal JNK and MAP kinase in the vasculature rather than in kidneys in Ang II-mediated hypertension. It may also be possible that renal p38MAP kinase and JNK are not influenced by processes linked to Na⁺/Mg²⁺ exchanger activity. Further investigation of tissue-specific Na⁺/Mg²⁺ exchanger-regulated MAP kinase activation should clarify these issues.

Since the Na⁺/Mg²⁺ exchanger has not yet been cloned or characterized, no selective inhibitors are currently available. For this reason, we used quinidine and imipramine, which inhibit enhanced vascular and renal MAP kinase activity that Na⁺/Mg²⁺ transport, is associated with increased oxidative stress [46], which may, in turn, influence the phosphorylation of p38MAP kinase and JNK. Although it is possible that improvement in blood pressure itself could contribute to normalization of MAP kinase activity, this is probably not the case in the present study, since phosphorylation of renal JNK and p38MAP kinase were not significantly altered in the treated groups, despite decreased blood pressure. These findings suggest that quinidine- and imipramine-sensitive processes are more important in the regulation of p38MAP kinase and JNK in the vasculature than in kidneys in Ang II-mediated hypertension.

The presence of secondary to activation of Na⁺-dependent Mg²⁺ transport, the role of Mg²⁺ in hypertension [48,49]. Nevertheless, based on our previous studies [28,29,32,33] as well as those of others [30,31], it is clear that Na⁺/Mg²⁺ exchanger activity is potently inhibited by quinidine and imipramine and that the effects observed in the present study could be mediated, at least in part, through changes in Na⁺ and Mg²⁺ transport. In conclusion, the present in vivo study demonstrates the novel findings that inhibitors of Na⁺/Mg²⁺ exchange activity attenuate development of hypertension and normalize enhanced vascular and renal MAP kinase activity in Ang II-induced hypertension. These data suggest that the Na⁺/Mg²⁺ exchanger could play a pathophysiological role in cellular processes contributing to elevated blood pressure in Ang II-dependent hypertension.

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