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Cross-talk between angiotensin II and glucagon receptor signaling mediates phosphorylation of mitogen-activated protein kinases ERK 1/2 in rat glomerular mesangial cells

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ABSTRACT

We have recently shown that the pancreatic hormone glucagon-induced phosphorylation of mitogen-activated protein (MAP) kinase ERK 1/2 as well as growth and proliferation of rat glomerular mesangial cells (MCs) via activation of cAMP-dependent protein kinase A (PKA)- and phospholipase C (PLC)/Ca^{2+}-mediated signaling pathways. Since circulating glucagon and tissue angiotensin II (Ang II) levels are inappropriately elevated in type 2 diabetes, we tested the hypothesis that glucagon induces phosphorylation of ERK 1/2 in MCs by interacting with Ang II receptor signaling. Stimulation of MCs by glucagon (10 nM) induced a marked increase in intracellular $[Ca^{2+}]_i$ that was abolished by [Des-His^1, Glu^9]-glucagon (1 μM), a selective glucagon receptor antagonist. Both glucagon and Ang II-induced ERK 1/2 phosphorylation (glucagon: $214 \pm 14\%$; Ang II: $174 \pm 16\%$; $p < 0.001$ versus control), and these responses were inhibited by the AT_1 receptor blocker losartan (glucagon + losartan: $77 \pm 14\%$; Ang II + losartan: $84 \pm 18\%$; $p < 0.01$ versus glucagon or Ang II) and the AT_2 receptor blocker PD 123319 (glucagon + PD: $78 \pm 7\%$; Ang II + PD: $87 \pm 7\%$; $p < 0.01$ versus glucagon or Ang II). Inhibition of cAMP-dependent PKA with H89 (1 μM) or PLC with U73122 (1 μM) also markedly attenuated the phosphorylation of ERK 1/2 induced by glucagon (glucagon + U73122: $109 \pm 15\%$; glucagon + H89: $113 \pm 16\%$; $p < 0.01$ versus glucagon) or Ang II (Ang II + U73122: $111 \pm 13\%$; Ang II + H89: $86 \pm 10\%$; $p < 0.01$ versus Ang II). Wortmannin (1 μM), a selective PI 3-kinase inhibitor, also blocked glucagon- or Ang II-induced ERK 1/2 phosphorylation. These results suggest that AT_1 receptor-activated cAMP-dependent PKA, PLC and PI 3-kinase signaling is involved in glucagon-induced MAP kinase ERK 1/2 phosphorylation in MCs. The inhibitory effect of PD 123319 on glucagon-induced ERK 1/2 phosphorylation further suggests that AT_2 receptors also play a similar role in this response.

1. Introduction

Diabetes mellitus is a major risk factor for the development of cardiovascular and renal target organ damage, leading to hypertension, atherosclerosis, ischemic heart disease and diabetic nephropathy [1,2]. Types 1 and 2 diabetes mellitus together affect more than 20 million Americans and rank as the sixth leading cause of disease-related death in the US. Type 1 diabetes is due to insulin deficiency, and therefore requires treatment with insulin or transplantation of pancreatic β cells

[3]. By contrast, the majority of the diabetic population has type 2 diabetes, which is not insulin-dependent and in most case does not require insulin therapy [1,2]. Type 2 diabetes is characterized by persistent hyperglycemia, impaired glucose tolerance, glomerular hyperfiltration, and progression of albuminuria, ultimately leading to renal injury [1,2]. Although increased insulin resistance rather than insulin deficiency plays a key role in the development of type 2 diabetes [1], the factors that contribute to increased insulin resistance remain incompletely understood.

The pancreatic hormone glucagon [4–6] and the vasoactive peptide angiotensin II (Ang II) [7–9] are two important factors implicated in the development of insulin resistance in type 2 diabetes. Clinical studies have shown that an inappropriate increase in the hyperglycemic hormone glucagon, not deficiency of the hypoglycemic hormone insulin, is the key characteristic of type 2 diabetes [1,10]. Glucagon binds to G_s protein-coupled receptors, activating glycogenolytic and gluconeogenic pathways to induce hyperglycemia through cAMP-dependent protein kinase A (PKA)- and phospholipase C/IP_3/Ca^{2+} signaling pathways [11–14]. Hyperglycemia promotes cell proliferation and growth (and/or hypertrophy) and induces tissue fibrosis by interacting with Ang II [15–17]. However, the role of glucagon in type 2 diabetic glomerular injury and the underlying cellular mechanisms are virtually unknown. By contrast, the role of Ang II in the development of diabetic nephropathy has been studied in humans using angiotensin-converting enzyme (ACE) inhibitors or Ang II type 1 receptor blockers [7–9]. There is evidence that the local renin–angiotensin system is activated in the kidney of diabetic animals and humans [18,19]. In addition to acting as a vasoactive hormone, Ang II is also a glycogenolytic and gluconeogenic peptide [20,21] as well as a growth factor and proliferative cytokine [22,23]. In the kidney, Ang II, acting via AT_1 receptors, causes contraction of vascular smooth muscle cells (VSMCs) and mesangial cells (MCs) and induces cell growth and proliferation through receptor-mediated PLC and cAMP signaling [22,24].

We have recently shown that glucagon stimulates MC growth and proliferation by inducing MAP kinase signaling via activation of cAMP-dependent PKA and phospholipase C [25]. However, we do not know whether these effects of glucagon involve interactions with Ang II or AT_1/AT_2 receptor signaling. Hyperglycemia has been shown to stimulate Ang II formation in MCs [19] and potentiate Ang II-induced growth and proliferation of VSMCs [15,17] and renal epithelial cells [16,26]. Glucagon may act on MCs either directly by activating its receptors or indirectly through hyperglycemia. We hypothesized that the pro-growth and proliferative effects of glucagon in MCs are mediated in part by receptor-activated MAP kinases ERK 1/2 and involve cross-talk or interactions with Ang II and/or its receptor signaling.

2. Materials and methods

2.1. Materials

Cultured glomerular MCs were obtained from American Type Culture Collection (ATCC). These cells are derived from the rat kidney and show both characteristics of glomerular MCs and typical responses to Ang II and glucagon [15,25,27]. RPMI-1640 medium, trypsin, heat-inactivated fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin) were purchased from ATCC. Human Val[5]-Ang II, glucagon and [Des-His[1]-Glu[9]] glucagon and Ang II enzyme immunoassay kits were obtained from Biochem/Peninsula Laboratories. cAMP enzyme immunoassay kits were purchased from R&D. The AT_1 receptor antagonist losartan was a gift from Merck Pharmaceuticals and the AT_2 receptor antagonist PD 123319 was donated by Pfizer. A rabbit polyclonal AT_1 receptor antibody targeting the N-terminal extracellular domain of the human AT_1 receptor (N-10) was purchased from Santa Cruz. Western blot supplies were obtained from Amersham. U73122, H-89 and wortmannin were purchased from Sigma.

2.2. Mesangial cell culture

Rat glomerular MCs were maintained in RPMI-1640 medium (10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 5 mM glucose, 1500 mg sodium bicarbonate/l; ATCC) containing 12% fetal calf serum (ATCC), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in 5% CO_2/95% O_2 as described previously [19,25,28]. MCs from passages 6 to 10 were sub-cultured in six-well plates containing the above growth medium until they reached 80% confluence, and maintained in serum-free medium for 24 h prior to the experiment. Unless otherwise specified, all experiments were performed with serum-free medium containing 5 mM glucose with or without glucagon or other blockers [19,25,28].

2.3. Measurement of intracellular calcium in MCs

To determine whether glucagon increases intracellular $[Ca^{2+}]_i$ by activating its specific receptors, we measured $[Ca^{2+}]_i$ responses to glucagon (10 nM) in MCs as described previously [14,25]. Sub-confluent MCs cultured on coverslips were loaded with Fura 2 (2 μM) for 30 min at 37 °C. After two washes with phosphate-buffered saline (PBS), coverslips were mounted on a perfusion chamber maintained at 37 °C, in turn mounted on a Nikon Eclipse TE 2000-U fluorescence microscope coupled with a Lambda DG4 illumination system (Sutter Instruments). Fura-2-loaded MCs were alternately excited at 340 and 380 nM every 3 s. Basal ratiometric calcium imaging was first recorded for 5–10 min with serum-free medium alone. MCs were then stimulated with glucagon (10 nM), and 340/380 ratiometric calcium images were continuously recorded at 3 s intervals for up to 10 min. A separate group of MCs was pretreated with the selective glucagon receptor antagonist [Des-His[1]-Glu[9]] glucagon (1 μM) for 30 min before glucagon stimulation. The effects of glucagon on $[Ca^{2+}]_i$ were determined by calculating the average magnitude of the peak $[Ca^{2+}]_i]_i$ responses during the entire glucagon stimulation (200 s) [14,25].

2.4. Western blots of total and phosphorylated ERK 1/2 in MCs

Animal and clinical studies have shown that hyperglucagonemia plays an important role in the development of glomerular hyperfiltration and mesangial cell injury [4–6,29]. In rats, we found that glomerular hyperfiltration induced by glucagon was attenuated by inhibiting angiotensin-converting

enzyme (ACE) with enalapril [30,31], together suggesting that glucagon interacts with Ang II to regulate glomerular filtration. To determine whether Ang II is involved in glucagon-induced phosphorylation of ERK 1/2 in MCs, sub-confluent MCs were treated with the ACE inhibitor captopril (10 μM) for 30–60 min before being stimulated with glucagon (1 nM). The specificity of glucagon-induced effects was verified using the glucagon receptor antagonist [Des-His[1]-Glu[9]]-glucagon (1 μM) [25,32].

After treatment, the medium was removed and MCs were washed twice with ice-cold PBS and lysed with RIPA buffer containing an inhibitor cocktail (Roche). Protein was measured using a BCA kit (Pierce, IL). A 10 μg protein from each sample was separated on 8–16% SDS/polyacrylamide gel and transferred semi-dry onto an Immobilon-P membrane (Millipore). Total and phosphorylated ERK 1/2 were detected by immunoblotting using a rabbit ERK 1/2 polyclonal antibody against the c-terminus of rat ERK 1/2 or a mouse anti-phosphorylated ERK 1/2 monoclonal antibody against an amino acid sequence containing phosphorylated Tyr-204 of human ERK 1/2 (Santa Cruz, CA) [25,33]. Western blot of β-actin (Sigma) was used to confirm equal protein loading. Immunoblots were visualized by enhanced chemiluminescence using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA) [15,25,33], scanned and analyzed using a microcomputer imaging device (MCID, Imaging Research, Ontario, CA).

2.5. Glucagon receptor-mediated increases in angiotensin II generation in MCs

Studies have shown that the local renin–angiotensin system, including renin, angiotensinogen and Ang II, is activated in the kidney of diabetic animals and humans [18,26]. Hyperglycemia purportedly plays a central role in this process by increasing Ang II generation especially in MCs [19,34]; however, it is not known whether glucagon can increase Ang II formation directly in MCs. Clinical studies in type 2 diabetes have found inappropriately elevated plasma glucagon levels, which may be responsible for persistent hyperglycemia and increased insulin resistance. To determine whether glucagon stimulates Ang II formation in MCs, sub-confluent cells were treated with serum-free medium alone, glucagon (10 nM), or glucagon plus [Des-His[1]-Glu[9]]-glucagon (1 μM) or captopril (10 μM) for 24 h at 37 °C. After treatment, the medium was removed and the cells first washed twice with ice-cold PBS, then twice with ice-cold acid buffer (5 mM acetic acid, 150 mM NaCl, pH 2.5) to remove any remaining cell membrane-bound Ang II [25,35]. Ang II was extracted from MCs in a buffer containing 20 mM Tris–HCl, 10 mM EDTA, 5 mM EGTA, 5 mM mercaptoethanol, 50 g/ml PMSF, 1 μg/ml aprotinin, and 1 μg/ml pepstatin, and measured using a sensitive and specific Ang II enzyme immunoassay kit (Biochem/Peninsula) [35]. Protein concentration in each sample was used to calculate final Ang II levels, expressed as pg/mg protein [35].

2.6. Role of AT_1 and AT_2 receptors in glucagon-induced ERK 1/2 phosphorylation in MCs

Glomerular MCs express both AT_1 and AT_2 receptors (primarily AT_1) and glucagon receptors [25,36]. In most cases, Ang II and glucagon stimulate MC growth and proliferation by activating AT_1 and glucagon receptors, respectively, whereas the role of AT_2 is not clear [22]. To determine their respective roles in mediating glucagon-induced ERK 1/2 phosphorylation, sub-confluent MCs were treated with serum-free medium alone, the AT_1 antagonist losartan (10 μM), the AT_2 antagonist PD 123319 (10 μM), or both AT_1 and AT_2 antagonists for 30 min before being stimulated with glucagon (1 nM) for 5 min. The antagonist(s) alone also served as a control. After treatment, MCs were washed, lysed and proteins extracted for Western blots of total and phosphorylated ERK 1/2 [25,33].

2.7. Role of AT_1 and AT_2 receptors in Ang II-induced ERK 1/2 phosphorylation in MCs

To determine whether Ang II induces ERK 1/2 phosphorylation in MCs by activating AT_1 and AT_2 signaling, sub-confluent MCs were treated with serum-free medium alone, losartan (10 μM), (PD 123319, 10 μM), or both losartan and PD 123319 for 30 min before being stimulated by Ang II (1 nM) for 5 min. After treatment, MCs were washed, lysed, and protein samples extracted for Western blots of total and phosphorylated ERK 1/2 [25,33].

2.8. Role of cAMP-dependent PKA, phospholipase C (PLC) and PI 3-kinase signaling in Ang II-induced ERK 1/2 phosphorylation in MCs

We recently showed that glucagon-induced ERK 1/2 phosphorylation involves activation of phospholipase C- and cAMP-dependent PKA signaling [25]. To determine whether activation of similar signaling pathways by Ang II induces ERK 1/2 phosphorylation, sub-confluent MCs were treated with serum-free medium alone ($n = 6$ wells), Ang II alone (1 nM), Ang II plus U73122 (1 μM), a selective PLC inhibitor, Ang II plus H-89 (1 μM), a selective PKA inhibitor, or the PI 3-kinase inhibitor wortmannin (1 μM) for 5 min. After treatment, protein samples were extracted for Western blot of total and phosphorylated ERK 1/2.

2.9. Statistical analysis

Where appropriate, results are expressed as mean ± S.E. Differences between groups were compared using one-way ANOVA followed by Dunnett's post hoc test. $P < 0.05$ was considered significant.

3. Results

3.1. Glucagon receptor-mediated increases in intracellular calcium ($[Ca^{2+}]_i$) in MCs

Glucagon activates its G_s protein-coupled receptors to increase $[Ca^{2+}]_i$ in hepatocytes and pancreatic cells [13,14], but its effect on $[Ca^{2+}]_i$ in MCs has only recently been reported [25]. Fig. 1 shows that under basal conditions, 340/380 ratiometric calcium imaging revealed faint $[Ca^{2+}]_i$ responses (panel A). Glucagon stimulation (10 nM) rapidly increased $[Ca^{2+}]_i$ in MCs (panel B). Pretreating MCs with a selective glucagon receptor antagonist, [Des-His[1]-Glu[9]]-glucagon (1 μM), effectively prevented glucagon-induced $[Ca^{2+}]_i$ responses, suggesting that the effect of glucagon was receptor-mediated.
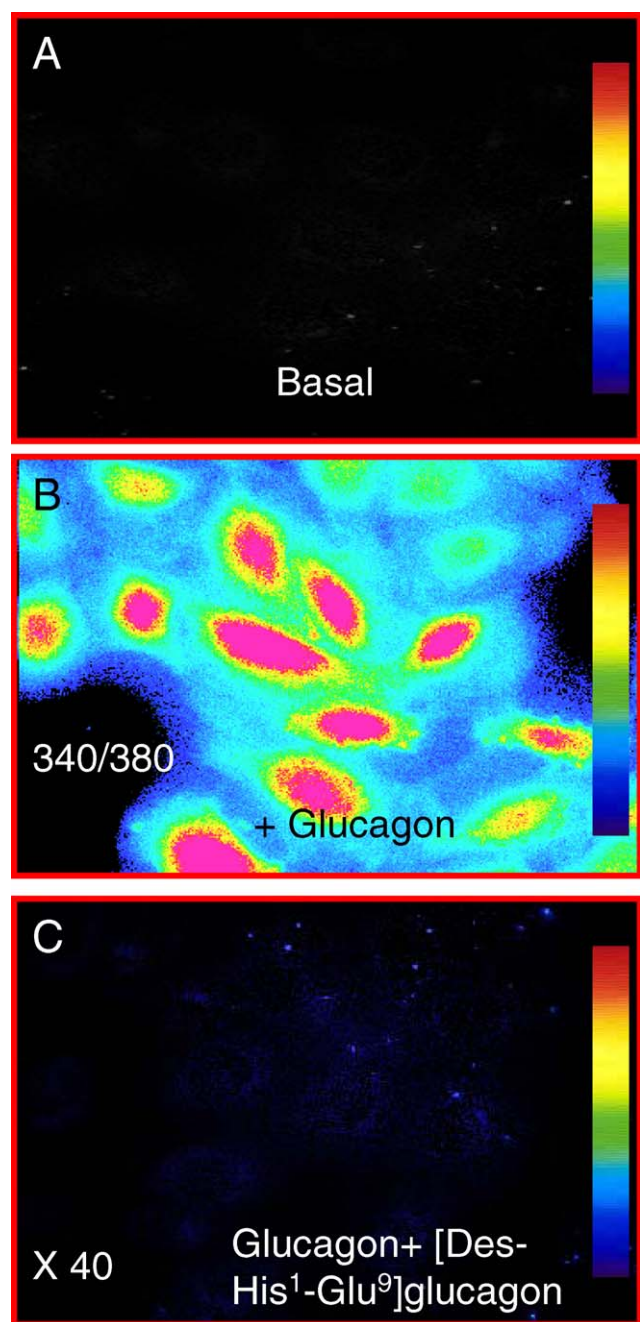
Fig. 1 – 340/380 Ratiometric calcium imaging shows intracellular calcium ($[Ca^{2+}]_i$) responses in MCs under basal conditions (panel A) and during glucagon stimulation (10 nM, panel B). The glucagon-induced increase in $[Ca^{2+}]_i$ was blocked by [Des-His^1,Glu^9] glucagon (1 μM, panel C). Intensity of $[Ca^{2+}]_i$ responses is shown as color bars, with red representing the highest level and dark blue as background. Magnification: 40×.

3.2. Effect of Ang II on glucagon-induced ERK 1/2 phosphorylation in MCs

As we reported recently [25], exposure of MCs to glucagon for 5 min almost doubled phosphorylated ERK 1/2 in MCs (185 ± 19%; $p < 0.001$ versus control), and this stimulatory
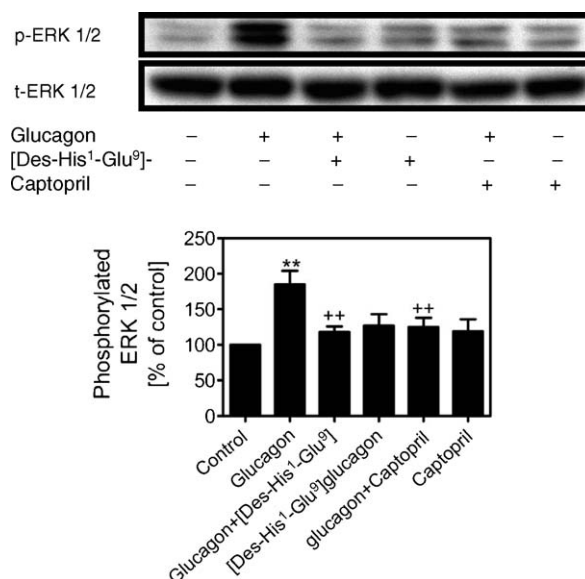
Fig. 2 – Effects of glucagon on MAP kinase ERK 1/2 phosphorylation in MCs. The top panel shows Western blots of phosphorylated and total ERK 1/2, and the bottom panel shows quantitative phosphorylated ERK 1/2 as percent of control. Glucagon-stimulated ERK 1/2 phosphorylation (1 nM) was blocked by [Des-His^1,Glu^9] glucagon (1 μM) and the ACE inhibitor captopril (10 μM), suggesting that the effect of glucagon is specific and involves interaction with Ang II. $^{**}p < 0.001$ vs. control; $^{++}p < 0.01$ vs. glucagon. $n = 4$–6 per treatment.

effect was blocked by the receptor antagonist [Des-His^1-Glu^9]-glucagon (1 μM) (118 ± 14%; $p < 0.01$ versus glucagon) (Fig. 2). We further determined whether glucagon induces ERK 1/2 phosphorylation by interacting with Ang II. Pretreating MCs with captopril to inhibit Ang II formation significantly attenuated glucagon-induced ERK 1/2 phosphorylation in MCs (125 ± 13%; $p < 0.01$ versus glucagon), whereas [Des-His^1-Glu^9]-glucagon (127 ± 12%; n.s.) or captopril alone (119 ± 17%; n.s.) had no significant effect. Total ERK 1/2 was not altered by any treatment.

3.3. Glucagon receptor-mediated increases in Ang II formation in MCs

High glucose has been shown to increase Ang II formation in MCs [19], but the effect of glucagon on Ang II formation in these cells has not been reported previously to our knowledge. Fig. 3 shows that basal Ang II was 788 ± 62 pg/mg protein, which was increased by 47% by glucagon (1160 ± 42 pg/mg protein; $p < 0.001$). Co-administration of glucagon with [Des-His^1-Glu^9]-glucagon (964 ± 38 pg/mg protein; $p < 0.01$) or captopril (854 ± 26 pg/mg protein; $p < 0.001$) significantly attenuated glucagon-increased Ang II formation.
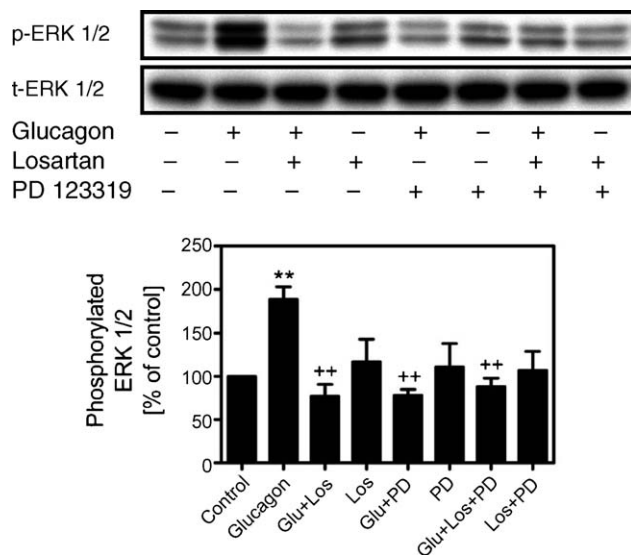
3.4. Role of AT$_1$ and AT$_2$ receptors in glucagon-induced ERK 1/2 phosphorylation in MCs

The effects of AT$_1$ or AT$_2$ receptor blockade on glucagon-induced ERK 1/2 phosphorylation have not been reported
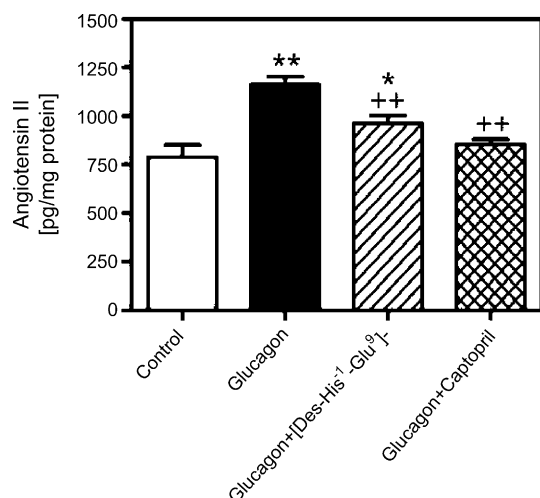
Fig. 3 – Effects of glucagon on angiotensin II (Ang II) generation in MCs. Treatment of MCs with glucagon (10 nM) for 24 h increased Ang II by 47%, and this effect was attenuated by [Des-His^1,Glu^9] glucagon (1 μM) and captopril (10 μM). $^*p < 0.05$, $^{**}p < 0.01$ vs. control; $^{++}p < 0.01$ vs. glucagon. $n = 6$ per treatment.

previously in any renal cells to our knowledge. Fig. 4 shows that glucagon-induced ERK 1/2 phosphorylation in MCs was markedly attenuated by the AT$_1$ antagonist losartan (glucagon: 189 14% versus glucagon + losartan: 77 ± 14%; $p < 0.001$)

Fig. 4 – Effects of Ang II AT$_1$ or AT$_2$ receptor blockade on glucagon-induced ERK 1/2 phosphorylation in MCs. The top panel shows Western blots of phosphorylated and total ERK 1/2, and the bottom panel shows quantitative phosphorylated ERK 1/2 as percent of control. The AT$_1$ receptor antagonist losartan (10 μM) and the AT$_2$ receptor antagonist PD 123319 (10 μM) both blocked ERK 1/2 phosphorylation induced by glucagon (1 nM). However, losartan and PD 123319 did not have an additive effect suggesting that their effects may be mediated by similar signaling pathways. $^{**}p < 0.01$ vs. control; $^{++}p < 0.01$ vs. glucagon. $n = 6$ per treatment.
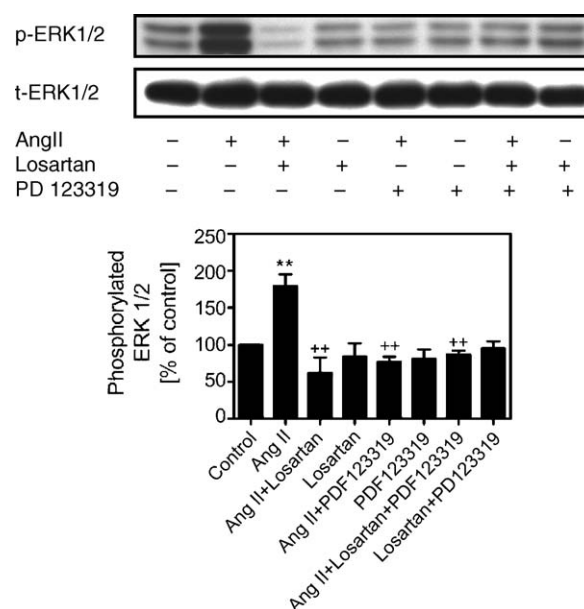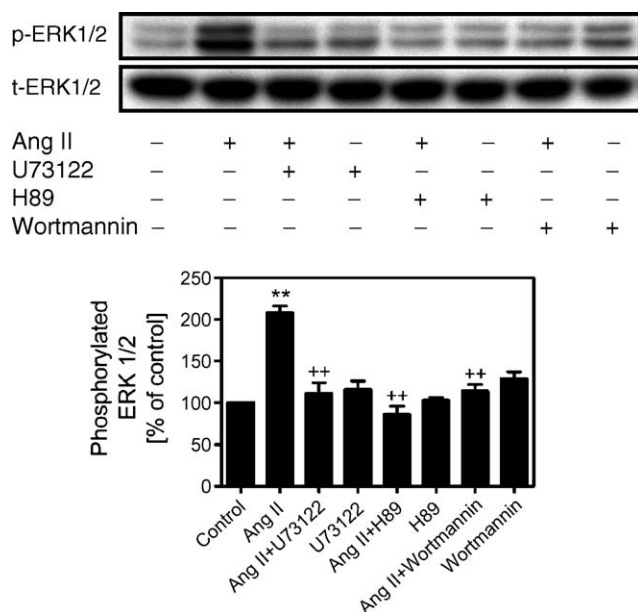

Fig. 5 – Effects of AT$_1$ and AT$_2$ receptor-mediated Ang II-induced ERK 1/2 phosphorylation in MCs. The top panel shows Western blots of phosphorylated and total ERK 1/2, and the bottom panel shows quantitative phosphorylated ERK 1/2 as percent of control. Ang II (1 nM) significantly increased phosphorylated ERK 1/2, and this response was completely blocked by pretreatment of MCs with losartan (10 μM) and PD 123319 (10 μM), respectively. $^{**}p < 0.001$ vs. control; $^{++}p < 0.01$ vs. Ang II. $n = 6$ per treatment.

or the AT$_2$ antagonist PD 123319 (78 ± 7%; $p < 0.001$ versus glucagon), suggesting that both AT$_1$ and AT$_2$ signaling are involved in glucagon-induced responses. Surprisingly, simultaneous blockade of AT$_1$ and AT$_2$ did not have an additive effect on glucagon-induced ERK 1/2 phosphorylation (107 ± 22%; n.s. versus control).

3.5. Role of AT$_1$ and AT$_2$ receptors in Ang II-induced ERK 1/2 phosphorylation in MCs

The effects of losartan and PD 123319 on glucagon-induced ERK 1/2 phosphorylation suggest cross-talk between Ang II and glucagon receptor signaling in MCs. Although Ang II is known to induce MC growth and proliferation, the mechanisms responsible remain unclear. Fig. 5 shows that like glucagon, Ang II also caused ERK 1/2 phosphorylation, resulting in nearly an 80% increase (179 ± 16%; $p < 0.001$ versus control). Blockade of AT$_1$ with losartan markedly attenuated the effect of Ang II below control (84 ± 18%; $p < 0.001$ versus Ang II), suggesting that AT$_1$ receptors may have a tonic influence in MCs. Surprisingly, blockade of AT$_2$ receptors with PD 123319 had similar inhibitory effects on Ang II-induced phosphorylation of ERK 1/2 (87 ± 7%; $p < 0.001$ versus Ang II). However, combined blockade of AT$_1$ and AT$_2$ had no additive effect on Ang II-induced ERK 1/2 phosphorylation (77 ± 5%; $p < 0.01$ versus Ang II). Losartan or PD 123319 alone did not alter either phosphorylated or total ERK 1/2.

Fig. 6 – Intracellular signaling mechanisms of Ang II-induced ERK 1/2 phosphorylation in MCs. The top panel shows Western blots of phosphorylated and total ERK 1/2, and the bottom panel shows quantitative phosphorylated ERK 1/2 as percent of control. Ang II-stimulated ERK 1/2 phosphorylation (1 nM) was significantly attenuated by pretreatment of MCs with the PLC-selective inhibitor U73122 (1 μM), the cAMP-dependent PKA inhibitor H-89 (1 μM), and the PI 3-kinase inhibitor wortmannin (1 μM), respectively. Inhibitor(s) alone had no effect on ERK 1/2 phosphorylation. $^{**}p < 0.001$ vs. control; $^{++}p < 0.01$ vs. Ang II. $n = 6$ per treatment.

3.6. Role of cAMP-dependent PKA, phospholipase C (PLC) and PI 3-kinase signaling in Ang II-induced ERK 1/2 phosphorylation in MCs

We recently found that glucagon-induced ERK 1/2 phosphorylation by receptor-activated cAMP-dependent PKA and PLC/IP$_3$/Ca^{2+} signaling [25]. Thus we questioned whether Ang II induces phosphorylation of ERK 1/2 by activating similar signaling transduction pathways. As shown in Fig. 6, blocking PLC with a selective inhibitor, U73122, significantly attenuated Ang II-increased phosphorylated ERK 1/2 (Ang II: 208 ± 8% versus Ang II + U73122: 111 ± 13%; $p < 0.01$). Blocking PKA with a selective inhibitor, H-89, likewise blunted the effect of Ang II (86 ± 10%; $p < 0.001$ versus Ang II). Furthermore, the PI 3-kinase-selective inhibitor wortmannin was also able to inhibit Ang II-induced ERK 1/2 phosphorylation (114 ± 8%; $p < 0.01$ versus Ang II). Since these inhibitors alone did not affect phosphorylated ERK 1/2, it would appear that Ang II can induce ERK 1/2 phosphorylation similarly via activation of cAMP-dependent PKA, PLC and PI 3-kinase signaling pathways.

4. Discussion

Activation of MAP kinase ERK 1/2 by vasoactive peptides, growth factors or proliferative cytokines such as Ang II and glucagon plays a critical role in promoting cell growth, differentiation, and proliferation during the development of target organ damage in diabetes and other cardiovascular and renal diseases [23,25,34,37,38]. Although Ang II and glucagon bind to different G protein-coupled receptors, their intracellular signaling pathways appear to converge or interact with each other [13,22]. The present study demonstrates that cross-talk between glucagon- and Ang II-induced MAP kinase ERK 1/2 phosphorylation in MCs involves at least two important mechanisms, stimulation of Ang II formation and activation of AT$_1$/AT$_2$ receptor-mediated signaling. This conclusion is derived from the observations that incubation of MCs with glucagon (10 nM) for 24 h increased Ang II by almost 50%, and that the ACE inhibitor captopril, the AT$_1$ antagonist losartan, and the AT$_2$ antagonist PD 123319 all significantly attenuated glucagon-induced ERK 1/2 phosphorylation in MCs. Moreover, both Ang II and glucagon increased ERK 1/2 phosphorylation through similar receptor-mediated signaling pathways, including PLC/IP$_3$/Ca^{2+}, cAMP-dependent PKA and PI 3-kinase activation. Thus our results suggest that glucagon interacts with Ang II and its receptor signaling to regulate MC function, including growth, hypertrophy and proliferation.

Although it is well documented that serum glucagon is inappropriately elevated in diabetes mellitus, especially type 2 (non-insulin-dependent) [4–6] and that the RAS is activated in the glomeruli of diabetic kidneys [7–9], whether glucagon interacts with Ang II and/or Ang II receptor signaling has not been explored previously to our knowledge. Glucagon is a 29-amino acid hormone synthesized in and released from pancreatic α cells [13]. Its primary action is to counter the glucose-lowering effect of insulin released from β cells to raise serum glucose levels. The hyperglycemic effect of glucagon is mediated by binding to GTP-binding heterotrimeric G$_s$ protein-coupled receptors, leading to activation of adenylate cyclase and increased intracellular cAMP production and PLC as well as mobilization of [Ca^{2+}]$_i$ [13,14,39]. There is evidence that the G$_i$ protein-activated signaling pathway may also play a role in glucagon-induced increases in [Ca^{2+}]$_i$ [13]. Activation of cAMP-dependent PKA and increases in [Ca^{2+}]$_i$ by glucagon are responsible for induction of MAP kinase ERK 1/2 phosphorylation, and hence cell growth, differentiation and proliferation. By contrast, the effects of Ang II are mediated by two receptors, AT$_1$ and AT$_2$ [22,36,40]. AT$_1$ Rs belong to the GPCR superfamily and is coupled via pertussis toxin-insensitive G proteins to activation of phospholipase C and calcium signaling [22]. However, AT$_1$ receptor is also coupled to other subunits of G proteins (G$_q$, G$_i$, G$_{q/11}$, etc.) to activate phospholipase D, phospholipase A$_2$, adenylate cyclase and ion channels [22], along with intracellular signaling that regulates gene transcription and/or expression of growth factors and proliferative cytokines that induce Ang II-induced target organ damage [22,23,40]. The precise nature and role of AT$_2$ receptor signaling are not well understood.

Although we have recently shown that glucagon can directly activate its G protein-coupled receptors to induce MAP kinase ERK 1/2 phosphorylation and growth and proliferation of MCs [25], our present results further suggest that these effects are mediated in part by stimulating Ang II

formation and/or cross-talk with Ang II receptors. Indeed, pretreatment of MCs with the ACE inhibitor captopril to block local Ang II formation significantly inhibited glucagon-induced ERK 1/2 phosphorylation (Fig. 2). This finding was further supported by the observation that exposure of MCs to glucagon for 24 h increased Ang II generation by 47%, which was blocked by captopril (Fig. 3). The Ang II-stimulating effect of glucagon is specific, since it was blocked by the glucagon receptor antagonist [Des-His^1-Glu^9] glucagon [25,32]. However, we did not address the question of how glucagon increases Ang II formation in the present study. Hyperglycemia (or high glucose) reportedly increases the expression of renin and its substrate angiotensinogen (and therefore Ang II formation) in MCs [18,19,34]. In the present study, MCs were maintained in serum-free medium containing normal glucose (5 mM) before and during treatment, so that this effect was not likely mediated via hyperglycemia. One plausible explanation is that glucagon activates adenylate cyclase to increase cAMP production, since cAMP itself is a powerful stimulator of renin expression in the kidney. Thus the cAMP signaling pathway may play an important role in this response.

The present study shows that blockade of AT$_1$ and/or AT$_2$ receptors with losartan and PD 123319, respectively, prevented glucagon-induced phosphorylation of ERK 1/2 in MCs. This is a surprising finding and it may be interpreted in three different ways. First, both AT$_1$ and AT$_2$ receptors are activated either separately or simultaneously in order to induce ERK 1/2 phosphorylation. Second, the AT$_1$ or AT$_2$ receptor antagonist is not selective for its respective receptor. Third, both AT$_1$ and AT$_2$ receptor antagonists block Ang II- and glucagon-induced ERK 1/2 phosphorylation by interfering not at the receptor site but at a distal level such as intracellular signaling pathways. In the present study, we had no evidence that either of losartan or PD 123319 interferes with glucagon receptor binding, or conversely that glucagon administration significantly increases AT$_1$ or AT$_2$ receptor protein expression (unpublished observations). The second explanation is unlikely since losartan and PD 123319 are perhaps the best characterized and widely used AT$_1$ or AT$_2$ receptor blocker, respectively [22]. We also have extensive experience in using losartan and PD 123319 to characterize and localize AT$_1$ and AT$_2$ receptors, respectively, in the kidney, adrenals, heart, and brain [41]. Therefore, one plausible mechanism that could explain the effects of Ang II receptor blockers is cross-talk between intracellular signaling pathways for glucagon and Ang II. This may include activation of cAMP-dependent PKA, PLC/IP$_3$/Ca^{2+} and PI 3-kinase signaling by both glucagon and Ang II. Glucagon receptors and Ang II AT$_1$ receptors are [Ca^{2+}]$_i$-mobilizing and cAMP-modulating G protein-coupled receptors (GPCRs), and their activation by agonist(s) induces effects that often mimic growth factor and/or proliferative cytokine actions on MAP kinase ERK 1/2 phosphorylation [14,22,38,42–44]. Although upstream MAP kinase activation by glucagon and Ang II may be different following binding to their respective GPCRs, activation of their downstream ERK 1/2 in MCs appears to involve similar intracellular signaling pathways, namely cAMP-dependent PKA, PLC and PI 3-kinase [13,22]. For example, in further studies we found that pretreatment of MCs with losartan (10 μM) or U73112

(a PLC-selective antagonist), not PD 123319, for 30 min significantly attenuated glucagon-induced increases in intracellular calcium (unpublished observations). In VSMCs, AT$_1$ receptor-activated MAP kinase ERK 1/2 phosphorylation may be mediated via a Gαq-PKC- or the Src-Ras-dependent mechanism, or via trans-activation of the epidermal growth factor receptor [22]. Whether glucagon-induced ERK 1/2 activation in MCs involves mechanisms similar to Ang II/ AT$_1$ receptors in VSMCs remains to be studied.

The effects of AT$_2$ receptor blockade with PD 123319 on Ang II- and glucagon-induced ERK 1/2 phosphorylation in MCs were somewhat unexpected. Although we found that the AT$_1$ receptor predominates in MCs with an AT$_1$/AT$_2$ receptor ratio of ∼80% versus ∼20%, the conventional view is that activation of AT$_2$ receptors opposes the classic actions of AT$_1$ receptors [22]. In the present study, PD 123319 blocked both glucagon- and Ang II-induced ERK 1/2 phosphorylation in MCs, suggesting that AT$_2$ receptors may take part in these responses in a similar manner to AT$_1$ receptors. Interestingly, we also found that PD 123319 blocked hyperglycemia-induced ERK 1/2 phosphorylation in MCs (unpublished observations). AT$_2$ has no effect on either intracellular [Ca^{2+}]$_i$ or cAMP, which are associated with classic activation of G$_q$ protein-coupled receptors or G$_s$/G$_i$ proteins, respectively [22]. Previous studies on AT$_2$ receptors in other types of cells have revealed diverse signaling pathways involving activation of serine/threonine phosphatases or phospholipase A$_2$ and changes in K$^+$ and T-type Ca^{2+} channels [22]. AT$_2$ may oppose AT$_1$-mediated MAP kinase activation by inducing dephosphorylation or inactivation of MAP kinase ERK 1/2 [22]. However, the effects of AT$_2$ on MAP kinase activation appear to depend on the specific type of cells or experimental conditions. For example, genetic deletion of AT$_2$ receptors was found to prevent [45], whereas overexpression of cardiac AT$_2$ receptors caused cardiac hypertrophy and heart failure in mice [46]. Furthermore, overexpression of AT$_2$ receptors has been shown to increase MAP kinase activity in VSMCs [22] and PC12W cells [47] and induce severe hypertrophy in neonatal cardiac myocytes [48]. In VSMCs or epithelial cells, AT$_2$ appears to induce nuclear factor-κB activation in a manner similar to AT$_1$ [40]. Collectively, the AT$_2$ receptor seems to positively regulate glucagon-, Ang II-, or hyperglycemia-induced activation of MAP kinase ERK 1/2, though the precise signaling mechanisms involved are not known.

In summary, the present study demonstrates that glucagon induces MAP kinase ERK 1/2 activation in part by stimulating local Ang II production and cross-talk with Ang II AT$_1$/AT$_2$ receptor-activated intracellular signaling pathways. The effects of glucagon were blocked by [Des-His^1-Glu^9] glucagon, a selective glucagon receptor antagonist, indicating a receptor-mediated event. These results are highly relevant to type 2 diabetic glomerular/MC injury, because proliferation, hypertrophy and mesangial expansion are hallmarks of early diabetic nephropathy. Since inappropriately elevated serum glucagon and activation of the intrarenal RAS are both seen during the development of type 2 diabetes, and since both glucagon and Ang II are glycogenolytic and gluconeogenic hormones that induce MAP kinase ERK 1/2 activation and promote growth and proliferation of MCs, we believe glucagon- and Ang II receptor-activated signaling pathways

may serve as preventive and therapeutic targets in type 2 diabetic nephropathy and other cardiovascular diseases.

Acknowledgments

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