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D1 dopamine receptor hyperphosphorylation in renal proximal tubules in hypertension

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A defect in the coupling of the D_1 receptor (D_1R) to its G protein/effector complex in renal proximal tubules plays a role in the pathogenesis of spontaneous hypertension. As there is no mutation of the D_1R gene in the spontaneously hypertensive rat (SHR), we tested the hypothesis that the coupling defect is associated with constitutive desensitization/phosphorylation of the D_1R. The following experiments were performed: (1) Cell culture and membrane preparations from rat kidneys and immortalized rat renal proximal tubule cells (RPTCs); (2) immunoprecipitation and immunoblotting; (3) cyclic adenosine 3′,5′ monophosphate and adenylyl cyclase assays; (4) immunofluorescence and confocal microscopy; (5) biotinylation of cell surface proteins; and (6) *in vitro* enzyme dephosphorylation. Basal serine-phosphorylated D_1Rs in renal proximal tubules, brush border membranes, and membranes from immortalized RPTCs were greater in SHRs (21.0 ± 1.5 density units, DU) than in normotensive rats (7.4 ± 2.9 DU). The increased basal serine phosphorylation of D_1Rs in SHRs was accompanied by decreased expression of D_1R at the cell surface, and decreased ability of a D_1-like receptor agonist (fenoldopam) to stimulate cyclic adenosine 3′,5′ monophosphate (cAMP) production. Increasing protein phosphatase 2A activity with protamine enhanced the ability of fenoldopam to stimulate cAMP accumulation ($17 \pm 4\%$) and alter D_1R cell surface expression in intact cells from SHRs. Alkaline phosphatase treatment of RPTC membranes decreased D_1R phosphorylation and enhanced fenoldopam stimulation of adenylyl cyclase activity ($26 \pm 6\%$) in SHRs. Uncoupling of the D_1R from its G protein/effector complex in renal proximal tubules in SHRs is caused, in part, by increased D_1R serine phosphorylation.

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Dopamine receptors in the central nervous system and peripheral tissues have been implicated in the regulation of blood pressure.[1,2] Dopamine exerts its action via two families of dopamine receptors that belong to the superfamily of G protein-coupled receptors (GPCR). The D_1-like receptor family (D_{1A} and D_{1B} in rodents, D_1 and D_5 in humans) is linked to stimulation of adenylyl cyclase, via the stimulatory G protein, $G\alpha_S$. The D_2-like receptor family (D_2, D_3, and D_4) is linked to inhibition of adenylyl cyclase, via the inhibitory G proteins, $G\alpha_i$ and G_O. There are remarkable parallels in the abnormal dopamine signaling, via D_1-like receptors, in rodent models of genetic hypertension (e.g., spontaneously hypertensive rat (SHR)) and human essential hypertension. Disruption of either one of the D_1-like receptors (D_1 or D_5) in mice leads to the development of hypertension.[3,4] D_1-like receptor inhibition of NHE3, Na^+/HCO_3^-, Cl^-/HCO_3^-, and Na^+/K^+ ATPase activities in the renal proximal tubule and medullary thick ascending limb of Henle is impaired in the SHR and Dahl salt-sensitive rat.[1,2,5–7] The impaired D_1-like receptor inhibitory effect on renal sodium transport in hypertension has been shown to be caused, in part, by impaired production of cytoplasmic second messengers.[1,2,5–7]

Despite the impairment of D_1-like receptors to exert their agonist effects in hypertension, there are no mutations in the coding region of the D_1R or D_5R in patients with essential hypertension or in genetically hypertensive rats.[8] Instead, in hypertension, the D_1R in the renal proximal tubule and medullary thick ascending limb of Henle is uncoupled from its G protein/effector complex,[5–10] a state that is associated with increased basal phosphorylation of the D_1R.[8,11] This finding is of interest because the functional status of dopamine receptors is determined, in part, by their state of phosphorylation, a process common to members of the GPCR superfamily.[12–14] Agonist occupation of GPCRs results in generation of second messengers, receptor phosphorylation, and desensitization causing a decrease in GPCR responsiveness to subsequent agonist stimulation.[13–15] Upon removal of the agonist, the attenuated responsiveness of the receptor is reversed by a dephosphorylation process subserved by protein phosphatases, probably of the 2A or 2B type, in the case of D_1-like receptors.[16–18] The dephosphorylation leads to cell surface membrane recycling and resensitization of GPCRs.

We have reported that the uncoupling of the D_1R in renal proximal tubules in the SHR may be a consequence of a defective resensitization process, caused by decreased activity of protein phosphatase$_{2A}$ (PP_{2A}).[16] However, the phosphorylation state and the cell surface membrane localization of the D_1R in kidneys of SHRs have not been reported. Therefore, the current studies were designed to determine the phosphorylation state and subcellular distribution of D_1 receptors (D_1Rs) in renal proximal tubules of WKY and SHRs. As the D_1R is phosphorylated at serine residues,[8,11,14,15,19,20] we measured the amount of serine-phosphorylated D_1Rs in renal cortical and brush border membranes (BBMs), and immortalized renal proximal tubule cells (RPTCs). In addition, we determined whether increasing protein phosphatase activity by pretreatment of cell membranes with the catalytic subunit of alkaline phosphatase[21,22] or increasing PP_{2A} activity in intact RPTCs, by protamine,[12,23,24] can enhance D_1R function in SHRs.

RESULTS

Phosphorylation of D_1Rs

To determine the extent of phosphorylation of the D_1R, *ex vivo* and *in vitro* studies were performed using freshly obtained rat kidney cortices, freshly prepared BBMs, and immortalized RPTCs because renal D_1Rs are predominantly located in proximal tubules.[1,7–11,16,25–31] Moreover, the D_1R defect in spontaneous hypertension has been well characterized in renal proximal tubules.[1–3,5,7–11,16,25–27,32] Basal levels of serine-phosphorylated D_1R were greater in renal cortical membranes, BBMs, and immortalized RPTCs from SHRs than from WKY rats (Figure 1, Table 1). The D_1-like agonist, fenoldopam, increased the quantity of serine-phosphorylated D_1R in WKY rats but not in SHRs (Figure 1, Table 1). Pretreatment with carbidopa (renal cortical membranes and BBMs) had no effect on basal serine phosphorylation of D_1Rs (not shown). Therefore, the results from the non-treated and treated groups were pooled together.

Distribution of D_1Rs in RPTCs

In order to determine the relative subcellular distribution of D_1Rs, we studied immortalized RPTCs in culture. Using live cells labeled with a cell-impermeant and non-cleavable biotin (sulfo-*N*-hydrosuccinamide (NHS)-biotin) reagent,[25,32] we found that the cell surface membrane fluorescence intensity was greater in WKY than in SHRs (Figure 2a). The cell surface membrane proteins (red) and D_1Rs (green) showed extensive colocalization (yellow) in cells from WKY rats, but were markedly reduced in cells from SHRs. Indeed, most of D_1Rs were internal to the cell surface membranes in SHRs.

We also compared the fluorescence intensity in permeabilized and non-permeabilized cells.

The D_1R can be visualized in non-permeabilized cells because the polyclonal D_1R antibody recognizes the third extracellular loop of the receptor. In non-permeabilized cells, fluorescence intensity was again greater in WKY than in SHRs (Figure 2b). However, after cell permeabilization, the total cellular expression of D_1Rs was similar in WKY and SHRs (Figure 2b), consistent with our previous report that the expression of D_1Rs, quantified by immunoblotting, in renal cortex or in RPTCs was similar in WKY and SHRs.[10]

To verify the visual impression of decreased cell surface membrane D_1Rs in RPTCs from SHRs, cells were cultured in Transwells to maintain apical and basolateral polarity. Cell surface membrane proteins were biotinylated with cell impermeant and non-cleavable sulfo-NHS-biotin,

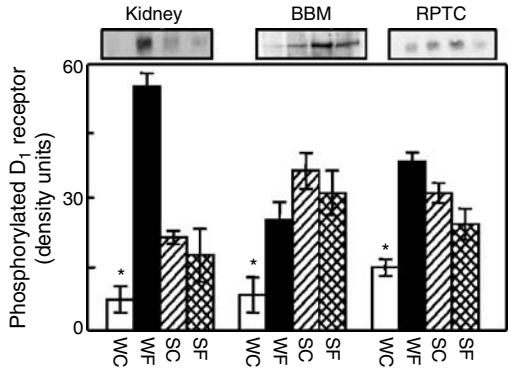

Figure 1 | Serine phosphorylation of D_1Rs in membranes from rat kidney cortex, brush border membranes (BBM) from rat kidney, and immortalized RPTCs from WKY and SHRs. Membranes were prepared as described in 'Supplementary text'. Membrane proteins were subjected to immunoprecipitation with monoclonal anti-phosphoserine antibodies and immunoblotting with polyclonal anti-D_1R antibodies as described in 'Materials and Methods'. The immunoreactive bands (≈70–80 kDa) were quantified by densitometry. WC and SC, controls from WKY and SHR cells, respectively; WF and SF, fenoldopam-treated cells from WKY and SHRs, respectively. Results are expressed as mean ± s.e.m., $n = 4$–5/group, $^*P < 0.05$ vs other groups except for cells from SHRs treated with D_1-like agonist, ANOVA, Newman–Keuls test. Inset is one of 4–5 independent immunoblots.

Table 1 | Serine-phosphorylated D_1Rs in cortical and BBMs from kidneys and immortalized RPTCs from WKY and SHRs were quantified by co-immunoprecipitation/immunoblotting as described in 'Materials and Methods'

	Kidney cortex		BBM		RPTC	
	WKY	SHR	WKY	SHR	WKY	SHR
Control/vehicle	7 ± 3*	21 ± 1.5	8 ± 4*	36 ± 4	14 ± 1.9*	31 ± 2.2
Fenoldopam (5 μM)	55 ± 3	17 ± 6	25 ± 4	31 ± 5	38 ± 2.1	24 ± 3.5

ANOVA Student—Newman–Keuls, $^*P < 0.05$, WKY control vs WKY fenoldopam-treated and SHR control, n=4–5.
The results in density units from Figure 1 are shown.
Abbreviations: BBMs, brush border membranes; D_1Rs, D_1 receptors; RPTC, renal proximal tubule cell.

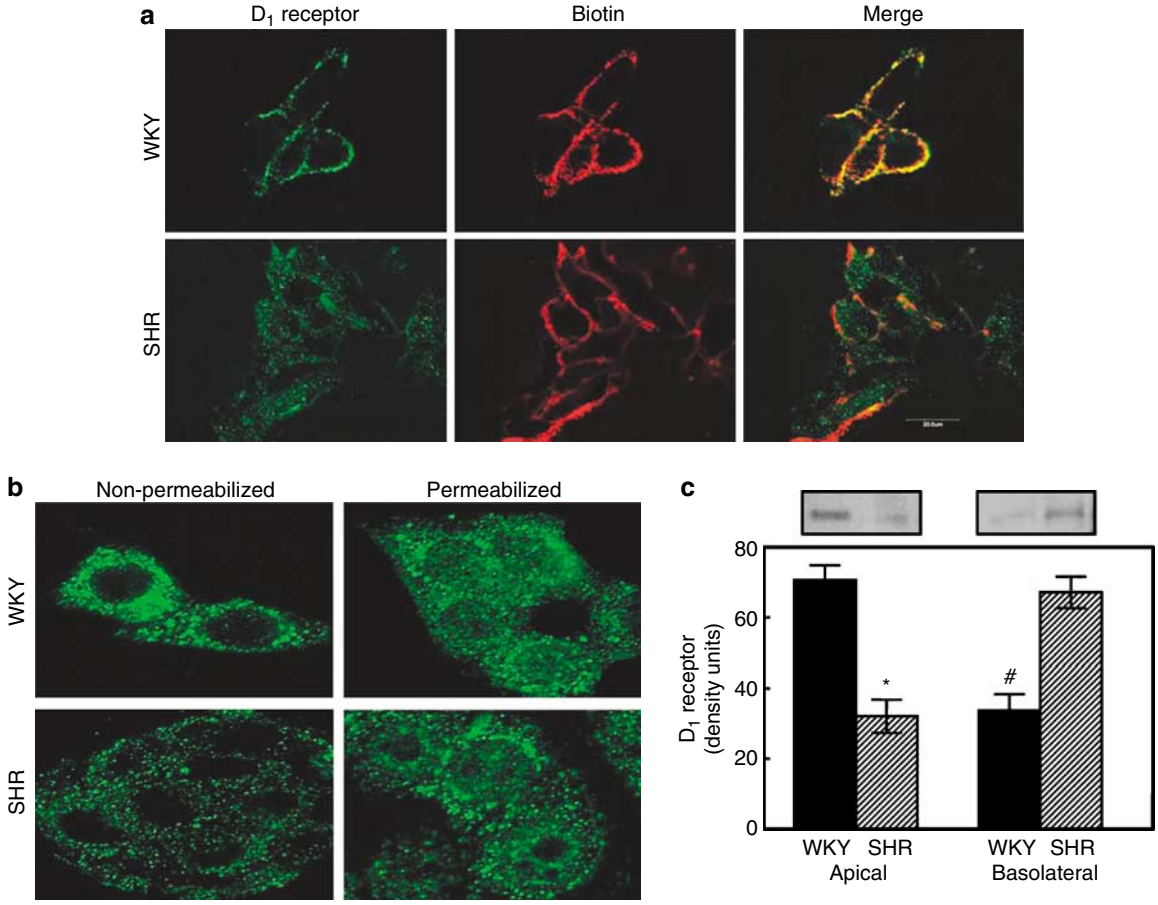

Figure 2 | Distribution of D_1Rs in immortalized RPTCs from WKY and SHRs. (**a**) Cellular distribution of D_1Rs in live cells. Cellular distribution was determined with cell-impermeant, non-clealvable biotin-labeling of live cells examined with double immunofluorescence confocal microscopy, as described in 'Materials and Methods'. Confocal images showed biotin-labeled membrane proteins as red color, and the D_1R as green color. The colocalization was seen as a yellow color in 'merge'. The image is one of at least three separate experiments. Bar = 20 μm. (**b**) Cellular distribution of D_1Rs by immunofluorescence. Non-permeabilized RPTCs from WKY rats had greater immunofluorescence than in SHRs. After permeabilization, fluorescence intensities were similar in the two rat strains. (**c**) Quantitation of D_1Rs on surface membranes of RPTCs from WKY and SHRs. The quantity of D_1Rs in apical and basolateral membranes was determined by biotinylation of proteins at the surface membranes of RPTCs grown on Transwells, as described in 'Materials and Methods'. D_1R density at the apical membranes was greater in WKY (67.8 $\pm$ 4.4 DU) than in SHRs (32.1 $\pm$ 4.7 DU). In contrast, D_1R density was greater at the basolateral membranes in SHRs (67.1 $\pm$ 4.5 DU) than in WKY rats (33.8 $\pm$ .4.6 DU) ($P < 0.01$, ANOVA Newman–Keuls test, $n = 5$). Inset is one immunoblot.

immunoprecipitated with anti-D_1R antibodies, and immunoblotted with avidin-conjugated peroxidase (Figure 2c). D_1R abundance at the apical membranes was greater in cells from WKY rats (67.8 $\pm$ 4.4 density units, DU) than those from SHRs (32.1 $\pm$ 4.7 DU) (Figure 2c), corroborating the immunofluorescence studies in Figure 2a and b. In contrast, D_1Rs were mainly located at basolateral membranes in cells from SHRs (67.1 $\pm$ 4.5 DU) relative to cells from WKY rats (33.8 $\pm$ .4.6 DU). Comparison of protein abundance in BBMs between WKY and SHRs is not precise because proteins can be redistributed within renal brush border microvilli[33] and D_1Rs are present in both brush border and subapical membranes.

We next studied the immunohistochemistry of rat kidney sections. We found that the cellular distribution of D_1Rs in renal cortical tubules was different between WKY and SHRs. In the renal cortex of WKY rats, D_1Rs were mainly located at microvillous brush border and apical membranes. In contrast, in SHRs, D_1Rs were distributed throughout the cytosol with minimal expression at brush border and apical membranes (Figure 3). Therefore, the kidney section studies confirmed the results obtained in immortalized RPTCs (Figure 2a, b and c). We also stained for rab GTPase 5 (Rab5), a marker protein for early or sorting endosome. Rab5-positive endosomes have been shown to serve as sorting compartments for both receptor recycling and degradation pathways.[34–36] Several studies have shown that internalized GPCRs, such as the β_2-adrenergic receptor, and the AT_1R, colocalize with Rab5 in endocytic vesicles.[35,36] We found that in the kidney cortex, Rab5 was distributed throughout the cytosol in both rat strains. However, some Rab5 expression was also observed in apical membranes in WKY. In contrast, the colocalization of D_1R with Rab5 in small vesicles (yellow color) in SHRs was mainly located at the basal membranes

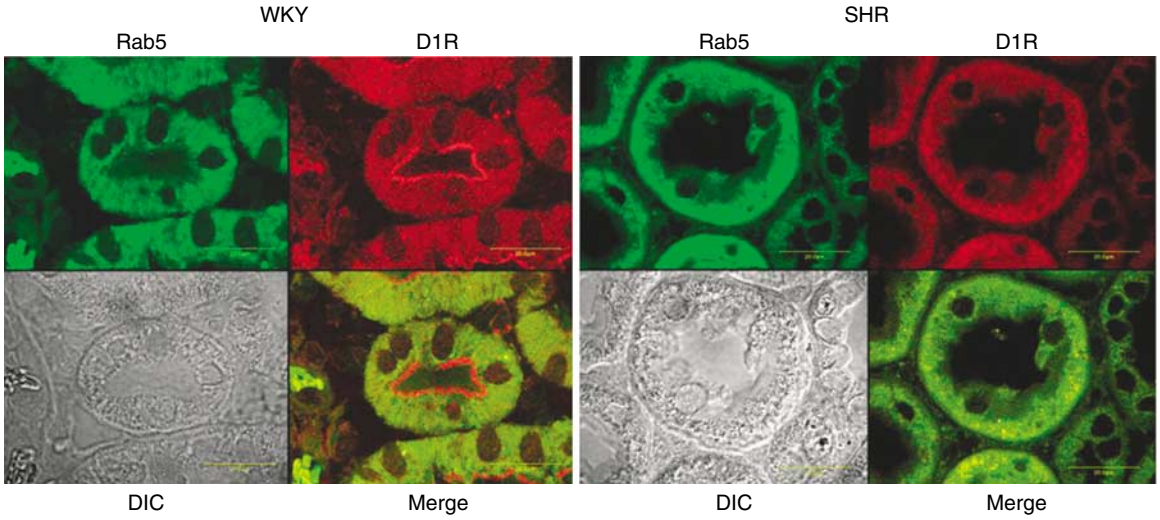

Figure 3 | Colocalization of D_1R with Rab5 in proximal tubules from rat kidney sections. Double immunofluorescence staining was performed as described in 'Materials and Methods'. The cellular distribution of D_1Rs in renal cortical tubules was different between WKY and SHRs. In WKY rats, D_1Rs (red color) were mainly located at microvillous brush border and apical membranes. In contrast, in SHRs, D_1Rs were distributed throughout the cytosol with minimal expression at brush border and apical membranes. Rab5 (green color) was distributed throughout the cytosol in both rat strains. However, some Rab5 expression was also observed in apical membranes in WKY. In contrast, in SHRs, the colocalization of D_1R with Rab5 in small vesicles (yellow color) was mainly located at the basal membranes. The image from transmitted light (DIC, differential interference contrast) is shown in gray. The image is one of the three separate experiments. Bar = 20 μm.

(Figure 3). The results indicated that more D_1Rs colocalized in Rab5-positive compartment vesicles in SHRs relative to WKY rats under basal conditions.

Effect of protamine on cAMP accumulation and distribution of D_1Rs

In preliminary studies, we found that the D_1R co-immunoprecipitated with PP_{2A} catalytic subunit (PP_{2A}c) but not with PP_1 or PP_{2B} in both WKY and SHRs (data not shown). The data indicated that PP_{2A} may directly interact with D_1R and that PP_{2A} may play a more important role than other protein phosphatases in D_1R regulation. PP_{2A} has been reported to be important in the dephosphorylation and resensitization of the D_1R.[16,17] Whereas, a D_1R agonist (fenoldopam) increased PP_{2A} activity in renal proximal tubules from WKY rats, PP_{2A} activity was decreased by fenoldopam in SHRs.[16] We next determined the consequences of protamine treatment on D_1R-mediated increase in cAMP accumulation and membrane expression of D_1Rs. Protamine has been shown to stimulate PP_{2A} activity but inhibit PP_1 activity.[12,23,24] We found that pretreatment of immortalized RPTCs with protamine overnight increased PP_{2A} activity by $77 \pm 5.5\%$ in WKY and $91.5 \pm 11.8\%$ in SHRs. Basal (control) cAMP levels in immortalized RPTCs were higher in WKY rats (282 ± 30 pmol/mg protein/20 min) than in SHRs (123 ± 18 pmol/mg protein/20 min, $n = 4$-5/group, $P < 0.05$ t-test). Protamine increased fenoldopam-induced cAMP accumulation in RPTCs from SHRs ($17 \pm 4\%$) (Figure 4a) to the same degree observed in RPTCs from WKY rats ($24 \pm 3\%$). These studies suggested that increasing PP_{2A} activity, and presumably the dephosphorylation of the D_1R, restored D_1R responsiveness to agonist stimulation in SHRs (see below).

Agonist-induced internalization of D_1R has been reported in many cell lines and types.[15,37,38] In our studies in RPTCs from WKY rats, fenoldopam stimulation resulted in more D_1R expression at the surface of one pole of the cell (observed under non-permeabilized conditions). The areas that lacked D_1R on the cell surface of non-permeabilized cells were probably the areas where the D_1R got internalized (with fenoldopam treatment) and therefore, could not be detected by fluorescence microscopy under non-permeabilized conditions. The D_1Rs were internalized by $64 \pm 4\%$ in RPTCs from WKY rats. In contrast, fenoldopam did not induce internalization of D_1Rs in RPTCs from SHRs ($1.0 \pm 1.1\%$, $P < 0.01$, $n = 3$) (Figure 4b). However, protamine pretreatment enabled fenoldopam to internalize more D_1Rs on the cell surface membranes of RPTCs from SHR ($45.5 \pm 5.8\%$) (Figure 4b), indicating that protamine allowed expression of D_1Rs on the cell surface that were then internalized with fenoldopam stimulation. It is also possible that recruitment of D_1Rs at one cell surface pole was increased by fenoldopam in WKY rats and that the recruitment was improved by protamine treatment in SHRs. Fenoldopam has been reported to increase the recruitment of D_1Rs to cell surface membranes of LLCPK1 cells.[39]

Effect of pretreatment of RPTC membranes with alkaline phosphatase on the ability of D_1-like receptor stimulation of adenylyl cyclase activity

To confirm the results obtained with protamine, we determined the effect of alkaline phosphatase treatment on adenylyl cyclase activity in RPTC membranes from SHRs. This method has been used to show the importance of dephosphorylation on the ability of natriuretic peptide receptor B to stimulate guanylyl cyclase activity.[22] Basal

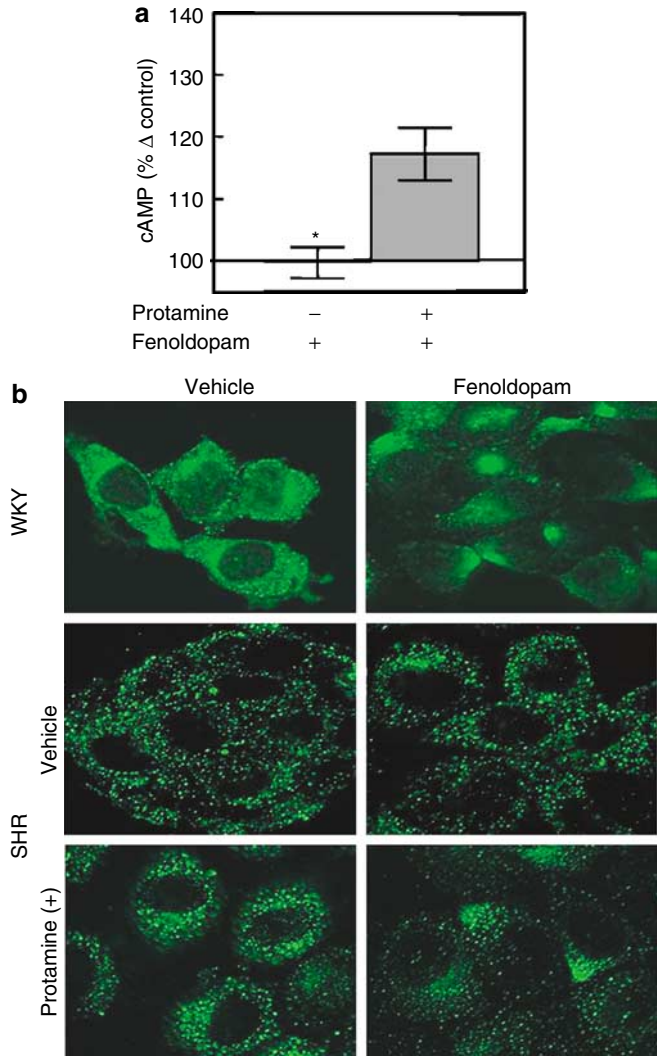

Figure 4 | Effect of protamine treatment on cAMP accumulation and cellular distribution of D_1Rs in immortalized RPTCs from WKY and SHRs. (**a**) Cells were treated with vehicle (control) or the D_1-like agonist, fenoldopam, in the presence or absence of protamine as described in 'Supplementary text'. In RPTCs from SHRs not treated with protamine, fenoldopam did not increase cAMP accumulation (-5.8 ± 4.1% increase from control). After protamine treatment, fenoldopam increased cAMP accumulation in RPTCs from SHRs ($+45 \pm 13$% to the same degree as those observed in RPTCs from WKY rats ($+64 \pm 21.1$%) ($n = 4$–5 group, $^*P < 0.05$ vs fenoldopam, paired *t*-test). (**b**) Effect of protamine on the cellular distribution of D_1Rs in RPTCs from SHRs. The cells, grown on coverslips, were treated with protamine as in (**a**), and immunofluorescence was studied under non-permeabilized conditions. Fenoldopam (5 μmol/l/10 min) increased D_1R expression at one pole of WKY cells (64 ± 4%, top panel) but not in SHR cells (1.0 ± 1.1%, $P < 0.01$, $n = 3$) (middle panel). Protamine treatment of SHR cells enabled fenoldopam to increase D_1R expression at one pole of SHR cells (bottom panel) (45.5 ± 5.8%). The image is one of at least three separate experiments. Bar = 10 μm.

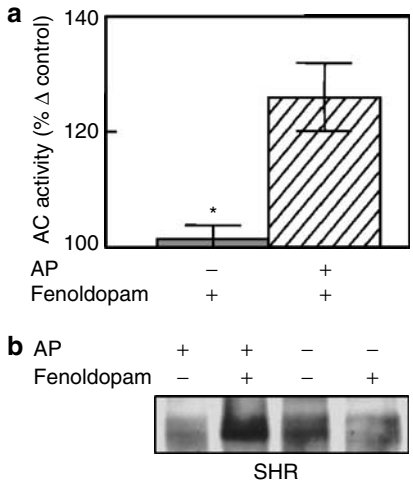

Figure 5 | Effect of alkaline phosphatase (AP) treatment on adenylyl cyclase activity and serine phosphorylation of D_1Rs in membranes of immortalized RPTCs from WKY and SHRs. (**a**) Effect of alkaline phosphatase on adenylyl cyclase activity. Membrane proteins from RPTCs were incubated with alkaline phosphatase as described as in 'Materials and Methods'. Fenoldopam (5 μmol/l/10 min) did not increase adenylyl cyclase activity in RPTCs from SHRs. Pretreatment of the membranes for 20 min with alkaline phosphatase enabled fenoldopam to increase adenylyl cyclase activity in RPTCs from SHRs ($^*P < 0.05$, vs fenoldopam, paired *t*-test, $n = 6$). (**b**) Effect of AP on D_1R phosphorylation in SHRs. Some of the RPTC membrane proteins from SHRs (Figure 4a) were lysed in TBSN buffer (see Materials and Methods) and subjected to immunoprecipitation with anti-phosphoserine antibody as in Figure 1. Alkaline phosphatase treatment decreased the basal phosphorylation of D_1Rs and increased the fenoldopam-induced phosphorylation of D_1Rs (similar to those noted in RPTCs from WKY rats – see Figure 1).

adenylyl cyclase activity was similar in RPTCs from SHRs and WKY (52.4 ± 8.0 and 55.5 ± 18.9 pmol/mg/min). (Note: the greater basal cAMP levels in intact RPTCs from WKY rats than in SHRs may be due to the fact that receptor recycling from the cytosol to the cell surface membranes could not occur in membranes.) Fenoldopam (5 μmol/l/10 min) increased adenylyl cyclase activity in RPTCs from WKY (50 ± 10% from control), but not in RPTCs from SHRs (1.0 ± 2.5%), consistent with previous reports.[10,16] A 20-min pretreatment of RPTCs with alkaline phosphatase decreased the basal adenylyl cyclase activity in membranes from RPTCs from SHRs (15.2 ± 2.3 pmol/mg/min, $P < 0.05$, paired *t*-test, $n = 6$). A purified catalytic subunit of PP_{2A} has been reported to decrease basal guanylyl cyclase activity.[22] It is possible that the decrease in basal adenylyl cyclase activity with alkaline phosphatase treatment may reflect a change in intrinsic activity of adenylyl cyclase (agonist independent activity).[37] In spite of the decrease in basal adenylyl cyclase activity, alkaline phosphatase treatment enhanced the ability of the D_1R agonist, fenoldopam, to stimulate adenylyl cyclase in RPTC membranes from SHRs (26 ± 6%) (Figure 5a). Thus, dephosphorylation of the hyperphosphorylated rat D_1Rs in RPTCs from SHRs (Figure 5b) restored the sensitivity of D_1Rs to agonist stimulation.

DISCUSSION

Desensitization of many GPCRs, defined as loss of responsiveness following agonist treatment, is thought to involve largely their phosphorylation by a variety of protein kinases, including G protein-coupled receptor kinase

(GRKs).[1,11,13–15,19,29,38,40] Agonist stimulation phosphorylates the D_1R at serine and threonine but not tyrosine residues.[8,11,14,15,19,38,40,41] In renal tissue, the serine phosphorylation of the D_1R is carried out by GRKs, mainly by GRK4, with minor contribution by GRK2, and resulting in its desensitization.[8,11,29] We have also reported that D_1R dephosphorylation, recycling to the cell surface membrane, and resensitization is abnormal in SHRs because of impaired activity of PP_{2A}.[16] Therefore, it is possible that the increased phosphorylation of the D_1R in RPTCs from SHRs is caused both by increased GRK activity and decreased PP_{2A} activity. The aim of this study was to determine the effect of dephosphorylation on D_1R subcellular localization and function in RPTCs from SHRs.

The quantity of phosphorylated D_1R in the basal state is greater in BBMs and renal cortex membrane, as well as in membranes of immortalized RPTCs, from SHRs than from WKY rats (Figure 1). This finding is in agreement with our studies in primary cultures of RPTCs from humans with essential hypertension compared with normotensive humans.[8,11] The similarity of the findings in the native kidney, primary culture of RPTCs in humans, and immortalized RPTCs in rats indicates that immortalization does not confer 'spurious' phenotypes, at least as far as the D_1R[5,7,9,10,16,25–27,29] and AT_1R are concerned.[42]

We now report that the cell surface membrane expression (determined morphologically and biochemically) of D_1Rs is decreased in immortalized RPTCs from SHRs relative to WKY rats (Figure 2c), in spite of similar quantities of D_1Rs in whole cells.[10] In the kidney of WKY rats, the D_1Rs are mainly located at the microvillous brush border and apical membranes, but less at the apical membranes in SHRs, consistent with the studies in immortalized RPTCs (Figure 2c). The increased colocalization of D_1R with Rab5 (Figure 3) in SHRs may reflect internalized D_1Rs that could not be recycled back to the apical membranes because of hyperphosphorylation of these receptors. The serine hyperphosphorylation of D_1Rs in SHRs accompanied by a decrease in expression at cell surface membranes, and an increase in colocalization with Rab5 may be taken to indicate that a majority of D_1Rs in renal proximal tubules are internalized and failed to be recycled to the cell surface membrane, resulting in defective function.[1,3,5–11,16] A similar process may explain the impaired renal D_1R function and high blood pressure associated with aging.[20]

We utilized pharmacological and biochemical approaches to determine whether the D_1R dysfunction in immortalized RPTCs of SHRs can be ameliorated. Protamine inhibits PP_1 activity but stimulates PP_{2A} activity by targeting the PP_{2A}-A subunit to interact with the PP_{2A} catalytic subunit.[12,23,24] A concentration of protamine that has been documented to increase PP_{2A} and decrease PP_1 activity increases D_1R density on cell surface membranes and responsiveness of D_1R to agonist stimulation in SHRs (Figure 3a and b). The protamine-induced change in D_1R expression may be the consequence of the resensitization of D_1Rs recruited from internalized receptors to a polar side of the cell surface. Fenoldopam, the D_1-like agonist used in our studies, has been reported to increase the recruitment of D_1Rs to the cell surface membrane of porcine renal cells, increasing D_1R responsiveness.[39]

To examine further the role of increased protein phosphorylation on D_1R dysfunction in SHRs, membranes from immortalized RPTCs were treated with alkaline phosphatase. Alkaline phosphatase has been used to study the role of dephosphorylation in GPCR function.[21] In the current studies, to measure adenylyl cyclase activity, membranes rather than intact cells were used. While increasing PP_{2A} activity with protamine in intact cells does not alter basal cAMP levels, basal adenylyl cyclase activity is decreased by the alkaline phosphatase treatment of RPTC membranes from SHRs. Nevertheless, alkaline phosphatase treatment of membranes from immortalized RPTCs from SHRs decreases the quantity of serine-phosphorylated D_1R (Figure 4b) and enhances the ability of a D_1R agonist to stimulate adenylyl cyclase activity (Figure 4a), in agreement with the results of the protamine studies. Taken together with the data using intact cells and protamine, these data strongly support the hypothesis that the hyperphosphorylation of the D_1R is responsible for the decreased D_1R activity in the SHR.

In summary, we have found increased basal levels of serine-phosphorylated D_1R in renal cortical membranes and BBMs and RPTCs from SHRs. This was associated with decreased levels of cell surface membrane D_1Rs (in SHRs), in spite of similar expression of D_1Rs in the whole RPTCs in WKY and SHRs. Increasing protein phosphatase activity, specifically PP_{2A}, increased D_1Rs on the cell surface membrane, enabled a D_1-like agonist to alter D_1R cell surface expression, and normalized D_1R linkage to adenylyl cyclase in the SHR. The cause of the impaired PP_{2A} action in SHRs is not known; however, we have reported that the regulatory PP_{2A} subunit, B56α, is not properly targeted to the renal proximal tubular cell membrane in SHR.[16] The cause of the mis-targeting of PP_{2A} B56α remains to be determined.

MATERIALS AND METHODS

Immunoprecipitation and immunoblotting

Immunoprecipitation of serine-phosphorylated D_1Rs. Membranes from kidney cortex, BBMs, and RPTCs (0.5–1 mg protein) were incubated with 4 μg anti-phosphoserine antibodies and protein G beads with rocking for 2 h or overnight in a cold room. The immune complexes on the beads were washed with homogenizing buffer, eluted with Laemmli buffer, and boiled for 10 min. The immunoprecipitates were then immunoblotted with polyclonal affinity-purified rabbit anti-rat D_1R antibody. The amount of protein transferred onto the nitrocellulose membrane was verified by Ponceau-S stain. All blots were visualized by ECL and quantified by densitometry, using Quantiscan, as reported.[10,16,25–27,29] All the data from different runs were normalized so that the sum of all density units from each run was 100%.

Biotinylation of apical and basolateral membrane proteins. The cells, cultured in Transwells, were washed with Dulbecco's modified Eagle's medium /F12-serum-free medium (SFM). Cell surface

membrane proteins were biotinylated[25,32] by adding a non-cleavable cell-impermeant biotin reagent (EZ-link sulfo-NHS-biotin) dissolved in SFM (500 μg/ml) to either the apical side (upper chamber) or basal side (lower chamber). The cells were incubated for 20 min on ice, washed, and lysed in phosphate-buffered saline containing 1% NP-40, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, and centrifuged 16 000 *g* for 10 min. The supernatants were subjected to immunoprecipitation with anti-D_1R antibody and immunoblotting with avidin-conjugated peroxidase.

Enzymatic dephosphorylation and adenylyl cyclase assay

For the dephosphorylation experiment and adenylyl cyclase assay, immortalized RPTCs from WKY and SHRs were used. The membranes were freshly prepared as described previously.[10,16,25] The membranes were resuspended in Tris and EDTA (TE) buffer containing 2 mM $MgCl_2$ (TEM buffer),[25] and subjected to enzymatic treatment with alkaline phosphatase.[21,22] The membranes (40 μg) were incubated with 5–10 units of alkaline phosphatase in 50 μl of reaction buffer (with the alkaline phosphatase kit) at room temperature for 20 min. Okadaic acid, a serine/threonine phosphatase inhibitor, was added (0.1 μmol/l) to stop the reaction. The membranes were assayed for adenylyl cyclase activity according to our published protocol.[28] Protein concentrations were determined using a protein assay kit.

Immunofluorescence microscopy

RPTC immunofluorescence staining was performed according to our previous report.[25] Briefly, RPTCs were grown on glass cover slips, fixed with 2% formaldehyde for 10 min at room temperature, and washed three times with phosphate-buffered saline. The cells were then stained under non-permeabilizing or permeabilizing conditions. Permeabilization was achieved by incubation with 0.1% Triton X-100 in phosphate-buffered saline buffer, for 10 min at room temperature. Some cells were pretreated with vehicle or protamine (5 μmol/l) (to increase PP_{2A} activity)[12,23,24] in complete culture medium for 24 h prior to treatment with fenoldopam. The cells were incubated anti-D_1R antibody (1:200) followed by Alexa 488-conjugated goat anti-rabbit secondary antibody (1:500). After extensive washing, the cover slips were mounted and the fluorescence densities and images were obtained using laser confocal scanning microscopy (Olympus Fluoview FV600) with original magnification $\times 60$ objective at an excitation wavelength of 488 nm; emission was detected at 505 nm. A total of 15 images from each cover slip ($n=3$) were counted for quantitation of receptor internalization.[43]

Immunohistochemical staining of rat kidney tissue

Rat kidney sections were prepared as described previously.[44] Briefly, the rat kidney was pre-flushed with an isotonic phosphate buffer and fixed with 2% glutaraldehyde through the aorta at a pressure of 140 mm Hg, and subsequently embedded in paraffin. Kidney sections were deparaffinized, rehydrated, permeabilized, and blocked with 5% goat serum. For D_1R colocalization with Rab5, the kidney sections were double-stained with specific polyclonal rabbit anti-rat D_1R and monoclonal anti-Rab5 antibodies, followed by Alexa 568-conjugated goat anti-rabbit and Alexa 488-conjugated goat anti-mouse secondary antibodies (1:500). The secondary antibodies also served as negative controls; no staining was observed using only the secondary antibodies (data not shown). The fluorescence images were obtained by laser scanning confocal microscopy, as described.[25]

Live cell labeling for confocal imaging

Cell surface membranes of live RPTCs grown on coverslips were labeled with EZ-link sulfo-NHS-biotin.[21,25,32] Live cells were washed once with Dulbecco's modified Eagle's medium/F12 SFM and biotin-labeled (500 μg/ml in SFM) for 20 min on ice.[25] The cells were washed three times with cold phosphate-buffered saline containing glycine (10 μmol/l), pH 7.0. The cells were then incubated for 10 min with avidin-conjugated Cy™ 5 (far red) (1:100 dilution in SFM) on ice. The cells were fixed and subjected to dual immunofluorescence staining with an anti-rat D_1R antibodies conjugated with Alexa-488. Cover slips were washed, mounted, and subjected to confocal microscopic study as above. For Cy™5, the excitation wavelength was 650 nm; emission was detected at 670 nm.

Statistical analyses

The data are expressed as means $\pm$ s.e. Comparison within groups was made using analysis of variance (ANOVA) for repeated measures (or paired *t*-test when only two groups were compared) and comparison among groups was made using factorial ANOVA with Neuman–Keuls test (or *t*-test when only two groups were compared). Corresponding periods between two different groups were analyzed using independent *t*-test. $P<0.05$ was considered significant.

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SUPPLEMENTARY MATERIAL

Supplementary text. Materials and References.

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