EXPERIMENTAL STUDY

High Glucose Stimulates Mineralocorticoid Receptor Transcriptional Activity Through the Protein Kinase C β Signaling

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Summary

Activation of mineralocorticoid receptor (MR) is shown in resistant hypertension including diabetes mellitus. Although protein kinase C (PKC) signaling is involved in the pathogenesis of diabetic complications, an association between PKC and MR is not known. Activation of PKCα and PKCβ by TPA (12-*O*-Tetradecanoylphorbol 13-acetate) increased MR proteins and its transcriptional activities in HEK293-MR cells. In contrast, a high glucose condition resulted in PKCβ but not PKCα activation, which is associated with elevation of MR protein levels and MR transcriptional activities. Reduction of endogenous PKCβ by siRNA decreased those levels. Interestingly, high glucose did not affect MR mRNA levels, but rather decreased ubiquitination of MR proteins. In db/db mice kidneys, levels of phosphorylated PKCβ2, MR and Sgk-1 proteins were elevated, and the administration of PKC inhibitor reversed these changes compared to db/+ mice. These data suggest that high glucose stimulates PKCβ signaling, which leads to MR stabilization and its transcriptional activities.

(Int Heart J 2017; 58: 794-802)

Key words: Diabetes mellitus, Diabetic complication, Resistant hypertension

According to several large-scale clinical trials including Randomized Aldactone Evaluation Study (RALES),[1] Eplerenone Post-Acute Myocardial Infarction Heart Failure (EPHESUS),[2] and Eplerenone in Mild Patients Hospitalization and Survival Study in Heart Failure (EMPHASIS-HF),[3] which have elucidated that add-on therapy of MR antagonist is beneficial for the improvement of prognosis in heart failure patients, we became aware in recent decades that aberrant MR activation could be crucially involved in cardiovascular morbidity and mortality. Indeed it is prevalently known that primary aldosteronism, in which aldosterone excess causes hypertension through MR activation, increases the risk of cardiovascular events such as myocardial infarction and arrhythmia by three- to five-fold over essential hypertension.[4] Additionally, add-on treatment of an MR antagonist has also been demonstrated to be effective in resistant hypertension (RHTN),[5] suggesting that activation of MR would play a key role on the progression of RHTN. These clinical findings has been driving many researchers to focus on the molecular mechanism of aberrant MR activation in RHTN and its related organ damages, but a detailed mechanism remains largely unknown.

Hypertension is approximately twice as frequent in diabetes mellitus (DM) compared with non-DM patients[6] and RHTN is highly associated with DM,[7] but the etiology of hypertension and cardiovascular complications in DM patients is not fully understood.[8] It was reported that add-on treatment of an MR antagonist alleviated persistent albuminuria in DM patients with conventional antihypertensive treatment,[9] which suggests that aberrant MR activation could be also an underlying mechanism of hypertension in DM patients. We have recently proposed such MR antagonist-responsive hypertension as "MR-associated hypertension"[10] and investigated the molecular actions of MR, among which we identified several novel coregulators of MR.[11,12]

DM is a major cause of macro- and microvascular complications. The molecular mechanisms for those vascular complications have been explained by several mo-

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This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (to H.S., I.K. and R.J.), Keio Gijuku Fukuzawa Memorial Fund for the Advancement of Education and Research (to H.S. and I.K.), and grant from the Smoking Research Foundation (to H.I. and H.S.).

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Received for publication December 27, 2016. Revised and accepted January 5, 2017.

Released in advance online on J-STAGE September 30, 2017.

doi: 10.1536/ihj.16-649

lecular signaling,[13] including the polyol pathway, hexosamine biosynthetic pathway, and the PKC pathway under high glucose conditions.[14-16] Sorbitol, which is accumulated by the polyol pathway, is crucial for the pathogenesis of diabetic neuropathy. The hexosamine biosynthetic pathway generates N-acetylglucosamine, which is followed by *O*-linked N-acetylglucosamine (*O*-GlcNAc) modification of many proteins. Our recent study showed that MR is modified by *O*-GlcNAc, thus resulting in the elevation of MR stability, which is followed by enhancing MR transcriptional activity (unpublished observation). The PKC pathway is also shown to be involved in diabetic cardiovascular complications as well as diabetic nephropathy.[16] PKC includes three subfamilies, that is, classical PKC, novel PKC, and atypical PKC, among which classical PKCs are well-documented to be involved in the pathogenesis of diabetic nephropathy.[17-20] Classical PKC mainly comprises PKCα and PKCβ. Hyperglycemia-induced activation of these PKCs exacerbates diabetic nephropathy through multiple mechanisms such as production of transforming growth factor β (TGFβ),[21] overactivation of vascular endothelial growth factor (VEGF),[22] and oxidative stress.[23] Involvement of MR activation in this context, however, remains unknown therefore in the present study we investigated the association between PKC signaling and MR activity in high glucose conditions. The present results showed that PKC® signaling plays an important role in the aberrant MR activation in diabetic pathophysiology.

Methods

All experimental protocols using recombinant DNA were approved by the Internal Review Board of Keio University School of medicine. All animal experiments were approved by the Institutional Animal Care and Use Committee and performed in accordance with the animal experimentation guidelines of Keio University School of Medicine.

Plasmid construct: 3×MRE-E1b-Luc were generous gifts from Dr. Bert W. O'Malley (Baylor College of Medicine, Houston). pcDNA MR (1-984) was subcloned into pGBKT7 vector as described previously.[11] pTB701-HA-PKCα, pTB701-HA-PKCβ were the generous gifts from Dr. Kuroda Shun'ichi (Osaka University). HA-PGL2-Basic-Luc and SS-PGL2-Basic-Luc were a generous gift from Dr. Lombès M (National Institute of Health and Medical Research (INSERM) (Institut National de la Santé et de la Recherche Médicale).

Chemicals: D-glucose and TPA (12-*O*-Tetradecanoylphorbol 13-acetate) were purchased from Sigma-Aldrich. Gö6976 (classical PKC inhibitor) and GF109203X (classical and novel PKC inhibitor) were purchased from merckmillipore. L-glucose was purchased from Tokyo chemical industry.

Antibody: Rabbit anti-MCR (H-300) antibody (SC-11412) and rabbit anti-human PKCβ2 (C-18) polyclonal antibody (SC-210) were obtained from Santa Cruz Biotechnology. Mouse anti-human MR monoclonal antibody (PP-H3122-00) was obtained from Perseus Proteomics Inc. Mouse anti-human α-tubulin antibody (DM1A) (CP06), rabbit anti-human phospho (ser657) PKCα antibody (06-822), rabbit anti-phospho serine antibody (AB 1603), and rabbit anti-human serum/glucocorticoid regulated kinase 1 (SGK1) monoclonal antibody (clone Y238) (04-1027) were obtained from merckmillipore. Rabbit anti-human PKCα polyclonal antibody (2056S) and rabbit anti-βactin antibody (4967) were obtained from Cell Signaling. Rabbit anti-human Phospho-PKCβ2 polyclonal antibody (11172-1) was obtained from Signalway Antibody Co. Ltd. Mouse anti-mono and poly ubiquitinated conjugates monoclonal antibody (HRP conjugate) (FK2H) (BML-PW0150) was obtained from Enzo Life Sciences. Anti-mouse IgG, HRP-linked whole antibody (NA931V) and anti-rabbit IgG, HRP-linked whole antibody (NA934 V) were obtained from GE Healthcare UK Ltd.

Cell culture: COS-7, HEK293, and HEK293-MR cells were routinely maintained in DMEM (life technologies) supplemented with 10% fetal bovine serum (life technologies). HEK293-MR cells stably expressing human MR have been established and described in detail previously.[11] Since cells expressing endogenous MR protein were hardly absent, we utilized stably expressing human MR cell. Even though it is an exogenous MR protein, we considered that the effect of protein modification after translation is equivalent.

RNA interference: COS-7, HEK293, and HEK293-MR cells were transfected with siRNAs, and reporter assays, western blots, and quantitative real time RT-PCR were performed as described previously.[11,24] Used siRNAs were listed below; PRKCA (s11092, Silencer select, Ambion), PRKCB (s11095, Silencer select, Ambion), Silencer negative control siRNA #1 (AM4611, Ambion).

Reporter assay: Twenty-four hours before transfection, 1 $\times 10^5$ cells per well of a 24-well dish were plated in the medium. All transfections were carried out by using lipofectamine LTX (life technologies) with 0.3 μg/well of the luciferase reporter, 0.01 μg/well of pRL-null internal control plasmids, and the indicated amounts of expression plasmids according to the manufacturer's instructions. siRNA transfection was carried out by using lipofectamine 2000 (life technologies) 24 hours before luciferase reporter transfection. Cell extracts were assayed for both Firefly and Renilla luciferase activities with a dual-luciferase reporter assay system (Promega). Relative luciferase activity was determined as ratio of Firefly/Renilla luciferase activities, and the results are shown as the mean (± SE) of triplicate values obtained from a representative experiment.

Western blots: The cells were lysed with TNE buffer including protease inhibitor cocktail (PIC) (Roche). In the assays detecting phosphorylated protein, phosphatase inhibitor cocktail (Roche) was added to the lysis buffer. Western blots were performed by corresponding antibodies as described before.[24]

Coimmunoprecipitation: The cells were lysed by RIPA buffer with PIC. In the assays detecting ubiquitinated protein, 10 mM *N*-Ethylmaleimide (NEM) was added to RIPA buffer. In the assays detecting phosphorylated protein, phosphatase inhibitor cocktail was added to RIPA buffer. After a 1 hour incubation with each antibody, protein G plus-agarose beads (Santa Cruz Biotechnology)

was subsequently added to the lysates and rotated overnight in the cold room. The beads were washed three times by 1 mL of Triton lysis buffer with PIC ±NEM ± phosphatase inhibitor cocktail. Then western blots were performed by corresponding antibodies as described above.

Quantitative real time RT-PCR: Total RNA was extracted using the RNeasy mini kit (QIAGEN), and concentration and purity of the RNA were checked spectroscopically using a Nanodrop spectrophotometer (Nano Drop Technologies). One μg of total RNA was reverse transcribed using TaqMan reverse transcription reagents. Primers for MR (Hs01031809_m1), SGK-1 (Hs00178612 _m1), ENaC (Hs00168906_m1), PKCα (Hs00925195_m 1), PKCβ2 (Hs00176998_m1), and βactin (Hs99999903_ m1) were purchased from Applied Biosystems. Quantitative PCR was performed using the ABI 7700 sequence detector (Applied Biosystems).

Animal experiment: Twelve-weeks-old db/+ and db/db male mice were obtained from Charles River Japan and db/+ mice were used as controls. The number of mice in each group was eight. One or five μg of Gö6976 or vehicle was administered intraperitoneally 7 days to inhibit PKC activity. Blood pressure, blood glucose, and body weight were measured before and after intraperitoneal administration. Sphygmomanometer used in this study was MK-2000ST (Muromachi Kikai Co., Ltd. Japan). After sacrificed, tissues were quickly frozen and stored at -80℃ to prepare for each assay.

Statistical analysis: We utilized a *t*-test or one way ANOVA for statistical analysis between the two groups. Variance of intended two groups was assayed by F test in advance, then a corresponding *t*-test was performed. We utilized two way ANOVA for statistical analysis between more than three groups. For the multiple comparison, we utilized Tukey's honestly significant difference as the post hoc analysis. All data are expressed as mean ± SE. $P < 0.05$ was considered statistically significant.

Results

PKC activation upregulated MR expression and transcriptional activity: We first examined whether TPA (12-*O*-Tetradecanoylphorbol 13-acetate), an activator for classical and novel PKCs, affect the MR levels and its transcriptional activities. In stably MR-expressing HEK293-MR cells, expression of MR protein was gradually decreased from 6 hours to 24 hours, whereas treatment with 100nM TPA increased MR protein levels with a peak at 12 hours (Figure 1A). The effects of TPA on MR protein levels were also shown in a concentration-dependent manner and co-treatment with Gö6976 (classical PKC inhibitor) or GF109203X (classical and novel PKC inhibitor) reversed TPA-induced upregulation of MR, suggesting that this upregulation of MR is due to activation of classical PKCs such as PKCα and PKCβ (Figure 1B). We next examined if TPA indeed activates PKCα and PKCβ. Western blot analysis showed that PKCα as well as PKCβ2 was phosphorylated by TPA treatment and the ratios of phosphorylated PKC/total PKC were elevated (Figure 1C). Not only chemical treatment modulating PKC activities but also the alteration of PKC expression changed MR protein levels. Overexpression of PKCα or PKCβ2, which was exogenously transfected using each plasmid, increased MR protein levels (Figure 1D). Reduction of endogenous PKCα or PKCβ2, which was introduced by corresponding siRNA, inhibited upregulation of MR protein by TPA (Figure 1E). Taken together with the above results, PKCα as well as PKCβ, especially PKCβ2, play a key role for the regulation of MR expression.

We next examined whether amounts of MR protein account for MR transcriptional activities. Reporter assays using the plasmid with 3×MRE (Mineralocorticoid Response Element) in the promoter indicated that treatment with 10 nM TPA markedly increased MR-mediated transcriptional activities by approximately five-fold, and this increment was attenuated by siRNA of both PKCα and PKCβ2 (Figure 1F). Endogenous *SGK1*, as a MR target gene, mRNA levels was also increased by TPA treatment, and this upregulation was remarkably suppressed by siRNA of both PKCα and PKCβ2 (Figure 1G). Taken together with data in Figure 1, we concluded that activation of PKCα as well as PKCβ pathway could increase MR protein expression and enhance MR transcriptional activities.

High glucose upregulated MR expression and transcriptional activity through PKCβ activation: As reported previously, PKCs are known to be activated in DM. Hence, we treated HEK293-MR cells with 30 mM D-glucose as high glucose (HG) condition and examined the effect of HG on MR activities through PKC activation. As a control, 5.6 mM D-glucose was treated as normal glucose (NG) condition. NG treatment was supplemented by 24.4 mM L-glucose, which is an enantiomer of D-glucose and cannot be utilized in human, to adjust the osmotic stress.

At first, we examined the activation of PKCs in HEK 293 cells under HG treatment. PKCβ2 phosphorylation was induced in HG treatment while PKCα phosphorylation was not changed, suggesting that PKCβ2 was preferentially activated by the HG condition (Figure 2A). Similar to TPA treatment, HG treatment increased MR protein levels in HEK293-MR cells, while MR expression gradually decreased in NG condition (Figure 2B). We next examined the effect of reduction of endogenous PKCs by siRNA on HG-induced upregulation of MR and found that only PKCβ2 siRNA significantly suppressed MR protein levels, and PKCα siRNA did not affect HG-induced MR upregulation (Figure 2C). In addition to the increase in MR expression, HG treatment also enhanced MR transcriptional activities. Reporter assays using the 3×MRE promoter indicated that HG treatment significantly increased MR-mediated transcriptional activities by approximately two-fold and this increment was lost by only siRNA of PKCβ2 but not of PKCα (Figure 2D). Endogenous MR target genes, *SGK1* and *ENaC*, were also upregulated in HG treatment and this upregulation was not attenuated by siRNA of PKCα, but PKCβ2 siRNA suppressed HG-induced elevation of MR target gene expression (Figure 2E). From these results, we concluded that HG treatment increased MR expression and activity, and this MR activation was exclusively mediated by

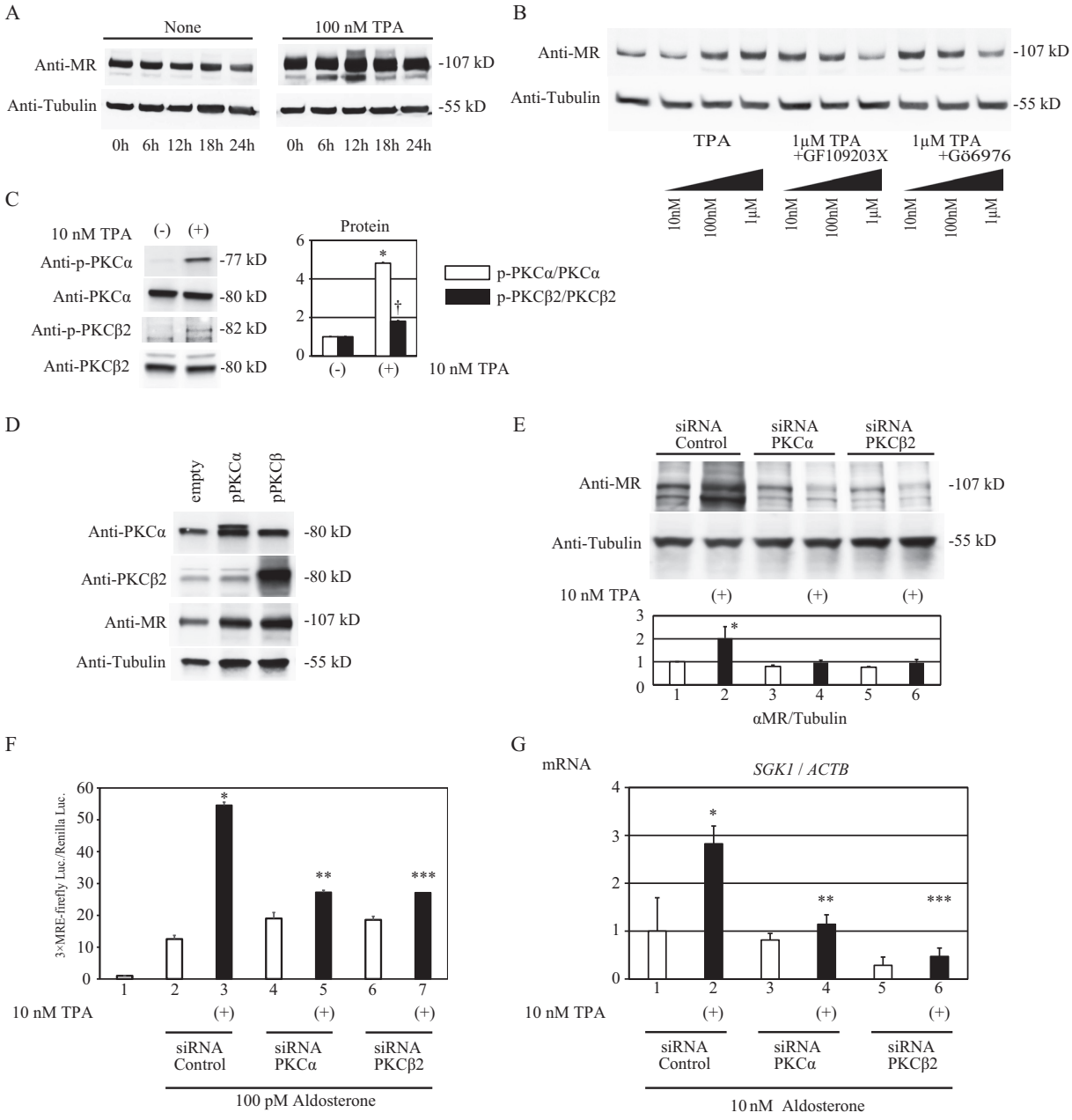

Figure 1. PKC activation upregulated MR expression and transcriptional activity. **A:** Time course changes of MR protein under PKC activator (TPA) treatment in HEK293-MR cells. **B:** Increase of MR protein was diminished by classical and novel PKC inhibitor (GF109203X) and classical PKC inhibitor (Gö6976). HEK293-MR cells. **C:** Validation of classical PKC activation in TPA treatment. $*P < 0.001$ versus TPA (-), $^{\dagger}P = 0.019$ versus TPA (-). HEK293-MR cells. **D:** Overexpression of PKCα and β increased MR protein. HEK293-MR cells. **E:** Increase of MR protein was diminished under knock down of PKCα and β by siRNA. Bar 2 $*P = 0.046$ versus siRNA control TPA (-) (bar 1). HEK293-MR cells. **F:** Reporter assays of MR transactivation. Bar 3 $*P < 0.001$ versus siRNA control TPA (-) (bar 2). Bar 5 $**P < 0.001$ versus siRNA control TPA (+) (bar 3). Bar 5 $**P = 0.003$ versus siRNA PKCα TPA (-) (bar 4). Bar 7 $***P < 0.001$ versus siRNA control TPA (+) (bar 3). Bar 7 $***P = 0.001$ versus siRNA PKCβ2 TPA (-) (bar 6). COS-7 cells. **G:** Levels of mRNA of endogenous *SGK1*. Bar 2 $*P < 0.001$ versus siRNA control (bar 1). Bar 4 $**P < 0.001$ versus siRNA control with TPA (bar 2). Bar 6 $***P < 0.001$ versus siRNA control with TPA (bar 2). ACTB stands for βactin. HEK293 cells.

PKCβ2 but not by PKCα.

As demonstrated above, HG treatment clearly increased MR protein levels, but *MR* mRNA expression was not altered in HG treatment (Figure 2E), suggesting that HG-induced MR upregulation would be processed post-transcriptionally. In addition to these results, we employed reporter plasmids comprising human MR promoter, which are known to mimic native gene regulation of MR,[25] and examined whether HG treatment had effect on transcriptional regulation of MR gene. HG treatment did not affect

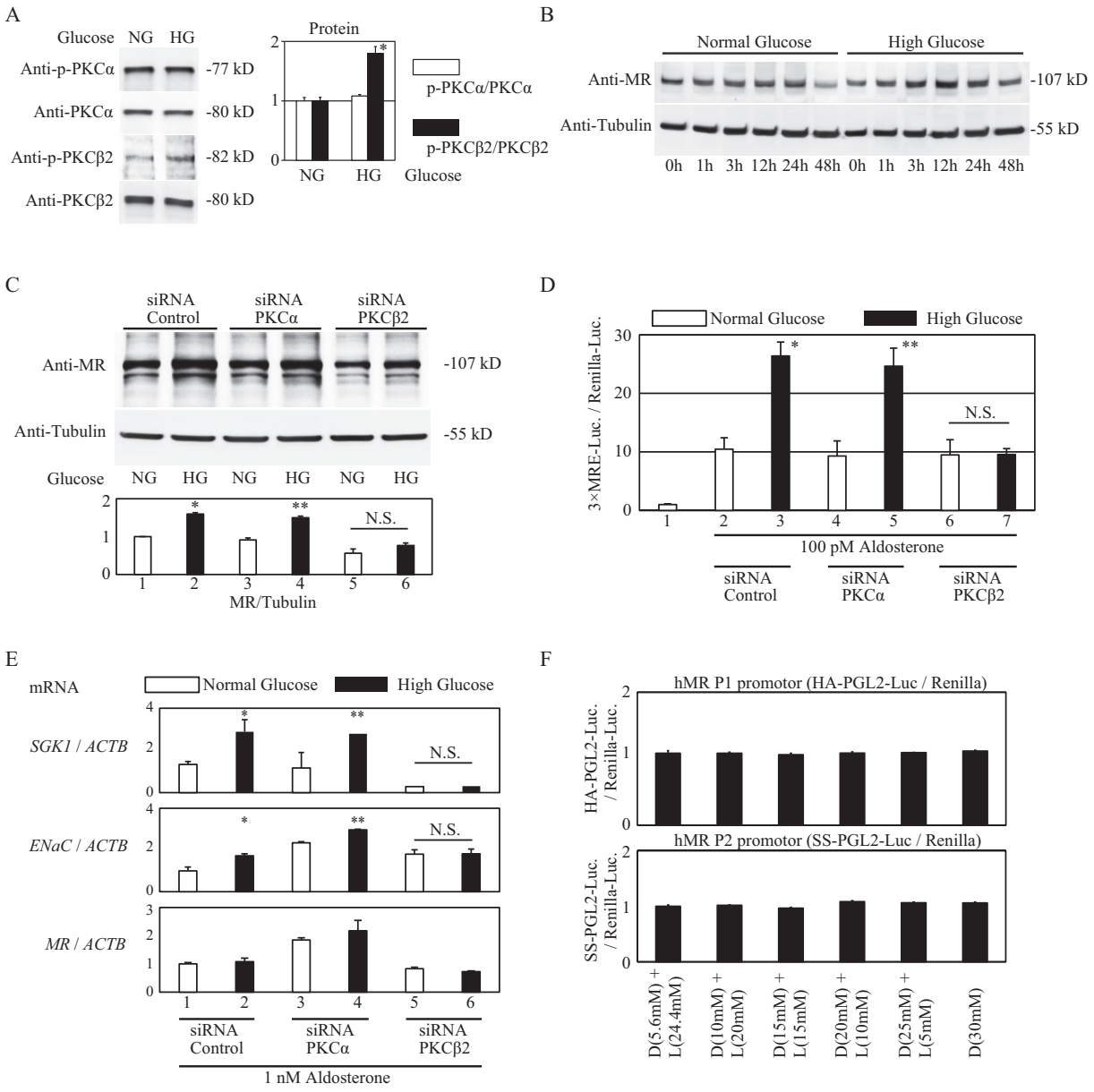

Figure 2. High glucose upregulated MR expression and transcriptional activity through PKCβ activation. **A:** Validation of PKCβ activation under high glucose (HG) treatment in HEK293-MR cells. *P = 0.004 versus normal glucose (NG). **B:** Time course changes of MR protein under HG treatment. HEK293-MR cells. **C:** Reduction of PKCβ, but not PKCα or control decreased levels of MR proteins. Bar 2 *P = 0.001 versus siRNA control with NG (bar 1). Bar 2 *P < 0.001 versus siRNA PKCβ2 with HG (bar 6). Bar 4 **P = 0.002 versus siRNA PKCα with NG (bar 3). Bar 4 **P < 0.001 versus siRNA PKCβ2 with HG (bar 6). HEK293-MR cells. **D:** Reporter assay of MR transactivation. Bar 3 *P < 0.001 versus siRNA control with NG (bar 2). Bar 3 *P < 0.001 versus siRNA PKCβ2 with HG (bar 7). Bar 5 **P < 0.001 versus siRNA PKCα with NG (bar 4). Bar 5 **P < 0.001 versus siRNA PKCβ2 with HG (bar 7). COS-7 cell. **E:** Levels of mRNA of endogenous *SGK1*, *ENaC,* and*MR*. *SGK1*; Bar 2 *P = 0.014 versus siRNA control with NG (bar 1). Bar 2 *P < 0.001 versus siRNA PKCβ2 with HG (bar 6). Bar 4 **P = 0.004 versus siRNA PKCα with NG (bar 3). Bar 4 **P < 0.001 versus PKCβ2 with HG (bar 6). *ENaC*; Bar 2 *P = 0.011 versus siRNA control with NG (bar 1). Bar 4 **P = 0.002 versus siRNA PKCα with NG (bar 3). *MR*; No significant differences. *ACTB* stands for βactin. HEK293-MR cells. **F:** Reporter assay of MR gene promoters (P1, P2). HEK293 cells.

P1 or P2 promoter of human MR (Figure 2F), indicating that MR increase in HG treatment might go through post-transcriptional modification.

HG induced MR phosphorylation and decreased ubiquitination of MR protein: PKC activation prompts kinase pathways, therefore we hypothesized that MR might be phosphorylated in the downstream of PKCs. An immunoprecipitation assay demonstrated that MR was more abundantly serine-phosphorylated in HG treatment (Figure 3A). In certain contexts, serine-phosphorylation competes with ubiquitin-dependent proteasomal degradation and stabilizes target protein. We therein hypothesized

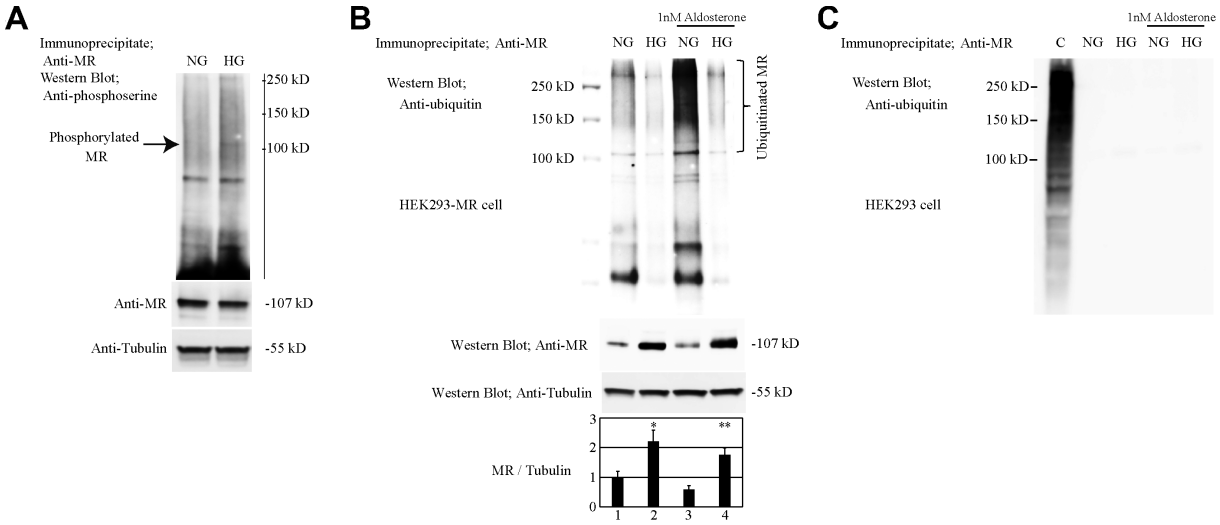

Figure 3. High glucose induced MR phosphorylation and decreased ubiquitination of MR protein. **A:** Phosphorylated MR protein under HG treatment. HEK293-MR cells. **B:** MR protein ubiquitination by aldosterone was diminished under HG treatment. Bar 2 **P* = 0.037 versus NG without aldosterone (bar1). Bar 4 ***P* = 0.045 versus NG with aldosterone (bar 3). HEK293-MR cells. **C:** Negative control of ubiquitination of MR. C; positive control, pcDNA-MR 1-984 was transfected in HEK293 cells. Others were HEK293 cell without transfected MR.

that HG-induced serine-phosphorylation of MR would counteract ubiquitination and increase stability of MR protein. As shown in Figure 3B, aldosterone treatment increased ubiquitination of MR and induced proteasomal degradation, but HG treatment remarkably decreased ubiquitination of MR leading to stabilization of MR protein. To validate the specificity of ubiquitinated MR signals, we performed the same experiment by using MR-negative HEK293 cells and found no signal detected in this condition (Figure 3C). These results indicated that posttranslational modification of MR protein, such as MR phosphorylation in HG treatment, might decrease aldosterone-induced ubiquitin-dependent proteasomal degradation and stabilize MR proteins, thus leading to the increase of MR expression and activity.

PKCβ-mediated MR activation was observed in db/db mice: According to the *in vitro* study above, we revealed that the HG condition could increase MR expression and activity through PKCβ activation. Then we examined it by using an *in vivo* model. db/db mice are known as a model of type 2 DM caused by a deficit of leptin receptor, so we examined whether MR activation would be observed in this model. First, we confirmed that blood glucose and blood pressure levels, as well as body weight, were expectedly higher in db/db mice (Figure 4A). After an intraperitoneal injection of PKC inhibitor, Gö6976, for 7 days, body weight and blood glucose were not changed (Figure 4A). In terms of blood pressure, Gö6976 treatment exhibited a significant fall in both db/db and db/+ mice, and the fall was more prominent in db/db mice, suggesting that the PKC pathway might have a greater impact on blood pressure regulation in db/db mice.

After sacrificing the mice, we collected kidney tissues and examined the expression levels of each aimed protein. To validate that PKCβ is activated in db/db mice, we examined phosphorylated PKCβ2 and PKCβ2 levels by western blot analysis. It was previously reported that hyperglycemia chronically increases the transcription level of PKCβ2, and the expression levels of PKCβ2 could be also a marker for activation of PKCβ2.[16,18,26] PKCβ2 and phospho-PKCβ2 expressions were increased in db/db mice (Figure 4B, C). PKCβ2 and phospho-PKCβ2 were expectedly decreased by Gö6976 treatment in db/db mice, while their expressions were unaltered by Gö6976 in db/+ mice (Figure 4B, C). In the meantime, PKCα and phospho-PKCα expressions appeared to be slightly increased in db/db mice, but the difference between db/db and db/+ mice was not statistically significant. In parallel with PKCβ2 and phospho-PKCβ2 expression, MR expressions were increased in db/db mice, and this increment was attenuated by Gö6976 treatment (Figure 4B, C). Levels of Sgk-1 protein, an MR target gene product, was also increased in db/db mice, and Gö6976 treatment decreased Sgk1 expression (Figure 4B, C). Taken together with the above *in vivo* experiment results, we concluded that MR expression, as well as MR activity, was upregulated in db/db mice, and this MR activation might be partly mediated by HG-induced PKCβ2 activation.

To explore the pathological significance of Gö6976 treatment, we also examined urinary albumin levels after a 7-day injection of Gö6976. The urinary albumin level was increased in db/db mice compared with db/+ mice, but Gö 6976 treatment failed to attenuate urinary albumin in db/db mice (data not shown). The applied dose of Gö6976 (1 μg/day) was supposed to be sufficient because high dose of Gö6976 (5 μg/day) had the similar potency on the suppression of MR expression (data not shown); therefore, we considered that the intervention period was not sufficient to gain the biological benefit.

Discussion

The present study demonstrated as a novel finding that classical PKC activation induces an increase of MR

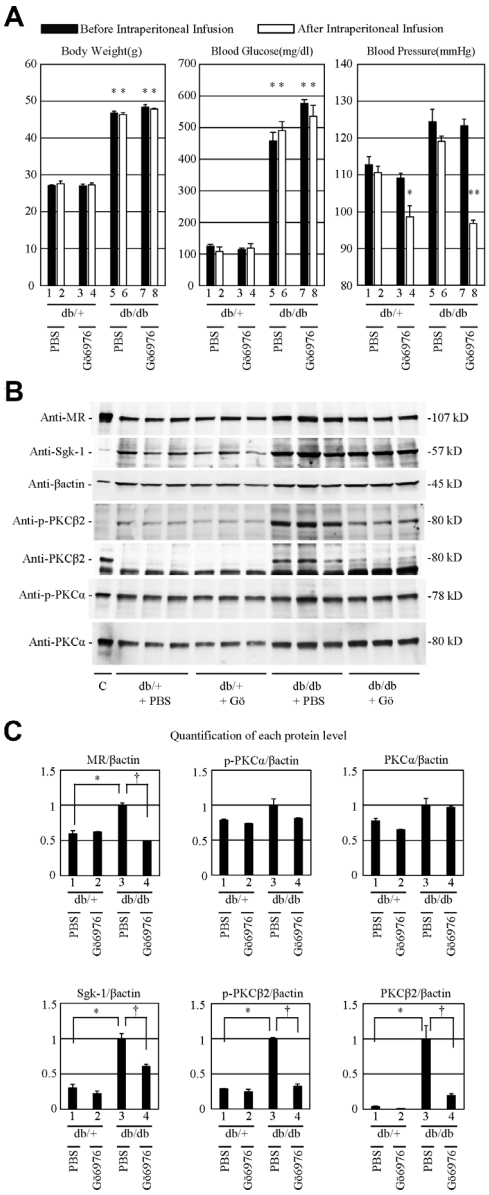

Figure 4. PKCβ-mediated MR activation was observed in db/db mice. **A:** Body weight (BW), blood glucose (BG) and systolic blood pressure (BP) before and after intraperitoneal infusion (i.p.) of classical PKC inhibitor (Gö6976) in *in vivo* study. BW; $P < 0.001$, db/+ mice (bar 1, 3) versus db/db (bar 5, 7). *$P < 0.001$ versus db/+ mice (bar 1 versus bar 5, bar 2 versus bar 6, bar 3 versus bar 7, bar 4 versus bar 8, respectively). BG; $P < 0.001$, db/+ mice (bar 1, 3) versus db/db mice (bar 5, 7). *$P < 0.001$ versus db/+ mice (bar 1 versus bar 5, bar 2 versus bar 6, bar 3 versus bar 7, bar 4 versus bar 8, respectively). BP; $P < 0.001$, db/+ mice (bar 1, 3) versus db/db mice (bar 5, 7). Bar 4 *$P = 0.002$ versus before i.p. (bar 3). Bar 8 **$P < 0.001$ versus before i.p. (bar 7). **B, C:** Protein levels of PKCβ2, phospho-PKCβ2, PKCα, phospho-PKCα, MR, and Sgk-1. MR; Bar 3 *$P < 0.001$ versus db/+ mice with PBS (bar 1). Bar 3 $^{\dagger}P < 0.001$ versus db/db mice with Gö6976 (bar 4). p-PKCα; No significant differences (bar 1 versus bar 3). PKCα; No significant differences (bar 1 versus bar 3). Sgk-1; Bar 3 *$P < 0.001$ versus db/+ mice with PBS (bar 1). Bar 3 $^{\dagger}P = 0.002$ versus db/db mice with Gö6976 (bar 4). p-PKCβ2; Bar 3 *$P < 0.001$ versus db/+ mice with PBS (bar 1). Bar 3 $^{\dagger}P < 0.001$ versus db/db mice with Gö6976 (bar 4). PKCβ2; Bar 3 *$P < 0.001$ versus db/+ mice with PBS (bar 1). Bar 3 $^{\dagger}P = 0.001$ versus db/db mice with Gö6976 (bar 4).

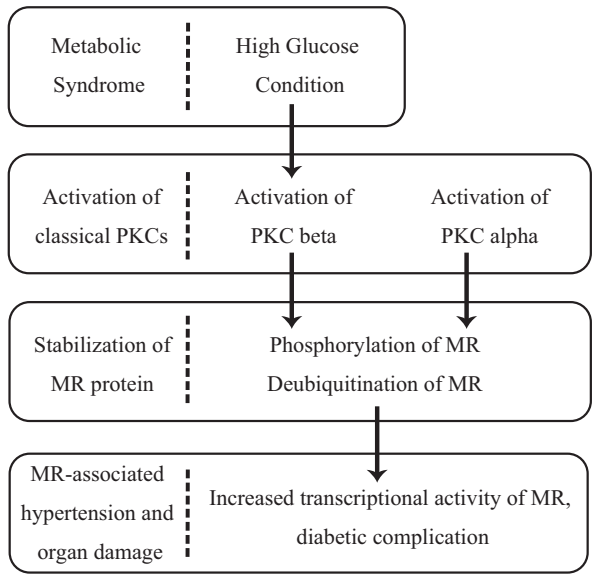

Figure 5. PKC signals and MR activation (summary).

proteins and enhances MR transcriptional activity. Additionally, HG treatment *in vitro*, as well as hyperglycemia *in vivo*, was involved in the upregulation of MR activity through PKCβ activation, whereas PKCα was not activated in our db/db mice. This result supports the notion that MR would be aberrantly activated in DM, which is consistent with the previous report that MR antagonist is really effective on the alleviation of albuminuria and other morbidities in DM.[27-29] In our db/db mice model, we failed to demonstrate that inhibition of PKCs could alleviate albuminuria, probably because the intervention period was not sufficient; so further studies would be needed in the future. A schematic summary of our findings in this study is shown in Figure 5.

In our *in vivo* experiments, inhibition of PKC by Gö 6976 decreased blood pressure in db/db as well as db/+ mice. It was previously reported that PKC activation could reduce NO availability in the endothelial function to relax vessels[30]; therefore, the blood pressure change in this study would be caused by multiple factors including the NO availability in addition to inhibition of MR function. Since some degree of PKC activation may occur in db/+ mice due to mild obesity, it is possible that administration of Gö6976 also decreased blood pressure even in these animals.

Our findings also implied that inhibition of the PKC pathway could be an alternative therapeutic approach to suppress the aberrant MR activity and its related cardiovascular events in DM. As it has been already suggested in a recent decade that PKCβ, especially PKCβ2, might play a key role in the progression of diabetic complications,[16] a selective PKCβ inhibitor, ruboxistaurin (Eli Lilly), was employed for large-scale clinical trials to prove the beneficial effects on the treatment for DM patients. This agent, however, failed to show significant superiorities over the control group in the prevention of diabetic complications.[31] According to the other clinical trials, a PKCβ inhibitor has some beneficial role in the prevention

against vision loss caused by diabetic retinopathy[32] and renal outcomes,[33] so there remains to be a controversy in this matter. Based on our results, we strongly recommend to relight the argument over the benefit of PKC inhibitors against diabetic complications. We demonstrated that not only PKCβ but also PKCα enhances MR activities, and therefore dual inhibition of PKCα and β might be more effective to give favorable outcomes. One of the recent reports, using streptozotocin-induced diabetic homozygous PKCα and β double knock-out mice, indicated that PKCα activation in glomeruli was associated with proteinuria, while PKCβ activation in renal tubules was associated with nephrosclerosis.[34] It was also reported that PKCα and other PKCs might be involved in the progression of diabetic complications.[16] Our study demonstrated that HG treatment preferentially induced PKCβ activation, which might lead to MR-associated hypertension and its related organ damages, but it should be extensively investigated in the future whether PKCα is activated in other diabetic animal models. Based on previous data, activation of PKCα and other PKCs might be involved in other diabetic models induced by streptozotocin, high fat diet, or gene manipulation. Free fatty acids are demonstrated to induce PKCα activation in certain contexts, which suggests that PKCα could be activated in obesity-induced diabetic models.[35,36] Further studies would be required to determine biological significance of PKCα on MR activation in DM.

As for molecular mechanisms of increased MR activity under the PKC activation, we clearly showed that MR protein levels are increased by pharmacological, as well as physiological PKC activation, which should contribute to enhanced transcriptional activity of MR. Deubiquitination of MR under HG treatment could account for increased stability of MR protein, which might be coupled with serine-phosphorylation of MR in the downstream of PKC activation. In general, activation of PKC signal is known to be linked to ubiquitination of proteins in various contexts.[37] Protein stability of Gadd45, a growth arrest and DNA-damage-inducible gene, is regulated by ubiquitin-proteasomal degradation. Activation of PKCδ signal counteracts this ubiquitination and increases protein stability of Gadd 45. In another context, PKCβ 2 inhibits ubiquitination-induced degradation of β-arrestin2.[38] In our study, it remains to be investigated which serine residue would be involved in the protein stability of MR under PKC signal activation. Bioinformatics approach for consensus phosphorylation motif of PKC suggests that there are many potential target residues in the molecule of MR therefore we should proceed to further studies in the future. Once the target residue of PKC signal in MR is identified, selective marker of the aberrant MR activation as well as more specific therapeutic approach could become available. Another possible mechanism of MR stabilization by PKC signals would be linked to the modulation of E3 ligase. It was reported that PKCβ2-induced protein stabilization of β-arrestin2 goes through the disruption of association between E3 ligase, Mdm2, and its target protein.[38]

There are several limitations in this study. PKCβ is generally considered to have two splice variants, namely PKCβ1 and β2. Antibodies and siRNA used in our study are specifically designed to recognize PKCβ2, and we did not elucidate the precise role of PKCβ1. It is possible that PKCβ1 is also involved in the process of MR activation induced by HG treatment, so that dual inhibition of PKCβ1 and β2 by ruboxistaurin could lead to unexpected outcome and selective PKCβ2 inhibitor, if any, might be an alternative solution to gain higher benefit over ruboxistaurin. Contribution of novel PKCs including PKCδ and PKCε, which were reported to drive DM-related morbidities, was not examined in our study either. We could speculate that several isotypes of PKC might be involved in the pathological activation of MR. The whole picture of the relation between MR and PKC signals would enable us to take an optimal risk management for cardiovascular events in DM patients.

Acknowledgments

Dr. Kuroda and Dr. Lombès have generously provided us several plasmids for this study.

Disclosures

Conflicts of interest: None.

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