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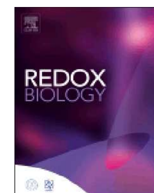
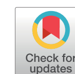


Redox-sensitive calcium/calmodulin-dependent protein kinase IIα in angiotensin II intra-neuronal signaling and hypertension



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ABSTRACT

Dysregulation of brain angiotensin II (AngII) signaling results in modulation of neuronal ion channel activity, an increase in neuronal firing, enhanced sympathoexcitation, and subsequently elevated blood pressure. Studies over the past two decades have shown that these AngII responses are mediated, in part, by reactive oxygen species (ROS). However, the redox-sensitive target(s) that are directly acted upon by these ROS to execute the AngII pathophysiological responses in neurons remain unclear. Calcium/calmodulin-dependent protein kinase II (CaMKII) is an AngII-activated intra-neuronal signaling protein, which has been suggested to be redox sensitive as overexpressing the antioxidant enzyme superoxide dismutase attenuates AngII-induced activation of CaMKII. Herein, we hypothesized that the neuronal isoform of CaMKII, CaMKII-alpha (CaMKIIα), is a redox-sensitive target of AngII, and that mutation of potentially redox-sensitive amino acids in CaMKIIα influences AngII-mediated intra-neuronal signaling and hypertension. Adenoviral vectors expressing wild-type mouse CaMKIIα (Ad.wtCaMKIIα) or mutant CaMKIIα (Ad.mutCaMKIIα) with C280A and M281V mutations were generated to overexpress either CaMKIIα isoform in mouse catecholaminergic cultured neurons (CATH.a) or in the brain subfornical organ (SFO) of hypertensive mice. Overexpressing wtCaMKIIα exacerbated AngII pathophysiological responses as observed by a potentiation of AngII-induced inhibition of voltage-gated K^+ current, enhanced *in vivo* pressor response following intracerebroventricular injection of AngII, and sensitization to chronic peripheral infusion of AngII resulting in a more rapid increase in blood pressure. In contrast, expressing the mutant CaMKIIα in CATH.a neurons or the SFO failed to intensify these AngII responses. Taken together, these data identify neuronal CaMKIIα as a redox-sensitive signaling protein that contributes to AngII-induced neuronal activation and hypertension.

1. Introduction

Elevated angiotensin II (AngII)-dependent signaling in the central nervous system has been implicated in the pathogenesis of several neuro-cardiovascular disorders, including hypertension and heart failure [1,2]. Numerous previous studies have shown that AngII signaling in central neurons leads to excessive influx of calcium, activation of NADPH oxidases causing increased production of reactive oxygen species (ROS), particularly superoxide ($O_2^{\bullet -}$), and regulation of voltage-gated potassium (K^+) channels [3–10]. Yin et al. demonstrated that the well-characterized AngII-mediated inhibition of outward K^+ current (I_{Kv}) is mediated by $O_2^{\bullet -}$ as overexpression of superoxide dismutases (SOD), which specifically scavenges $O_2^{\bullet -}$, completely abolishes this AngII-response [8]. Several studies by Sumners, Raizada, and colleagues have demonstrated that the AngII-mediated reduction in I_{KV} and increase in neuronal firing rate also involves specific kinases, such as calcium/calmodulin-dependent protein kinase II (CaMKII) [6,9]. However, the precise signaling mechanism(s) by which ROS and these kinases communicate with each other, if at all, to alter neuronal ion channel activity, neuronal activation, and ultimately an increase in systemic blood pressure remain unknown.

CaMKII is a multifunctional enzyme which couples increases in intracellular Ca^{2+} to regulation of ion channels, transcription of genes and apoptosis [11–13]. Under resting conditions CaMKII remains inactive, but upon binding with Ca^{2+}/CaM the autoinhibitory regulatory domain of CaMKII is relieved from the kinase domain causing CaMKII activation. Autophosphorylation of a threonine residue (T286 in CaMKIIα) within the autoinhibitory region of CaMKII then leads to a Ca^{2+}/

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CaM independent CaMKII activity by preventing the reassociation of the autoinhibitory region with the kinase domain. Studies have shown that AngII-mediated pathological responses in the heart involve a second Ca^{2+}/CaM independent activity regulated by pro-oxidant conditions [12,14]. More specifically, Erickson et al. have demonstrated that AngII-generated ROS oxidize paired methionine residues (M281/282) in CaMKII-delta (CaMKIIδ), the cardiomyocyte-localized CaMKII isoform, leading to sustained CaMKII activity and contributing to the pathogenesis of cardiac disease [12]. Interestingly, and of particular relevance to the current study, the paired methionines (M281/282) in CaMKIIδ are replaced by a cysteine (C280) neighboring a methionine (M281) in the neuronal CaMKIIα isoform. Considering cysteine, like methionine, contains a redox-sensitive thiol, we hypothesize that C280 and M281 in CaMKIIα are susceptible to ROS-mediated post-translational modification.

Within the brain, AngII acts on specific nuclei that are important in autonomic control of cardiovascular function, including the paraventricular nucleus (PVN) [16], rostral ventrolateral medulla (RVLM) [17], and nucleus tractus solitarius (NTS). AngII can also activate neurons located in the blood-brain barrier deficient circumventricular organs, such as the subfornical organ (SFO) [18], resulting in enhanced sympathoexcitation. Increased sympathetic output is known to contribute to hypertensive symptoms, including increased vasoconstriction, enhanced salt and water reabsorption, increased heart rate, and activation of T-lymphocytes and inflammatory cytokines [19–23]. Past studies have also demonstrated that scavenging $O_2^{\bullet-}$ with adenoviral-mediated overexpression of CuZnSOD (AdCuZnSOD) in the SFO attenuates blood pressure in an AngII-infused hypertensive animal model [4]. However, the role of downstream effector molecules that may be sensitive to changes in the redox environment, such as CaMKIIα, in the brain in mediating the AngII-induced hypertensive response is unclear.

In the present study, we tested the hypothesis that C280 and M281 in CaMKIIα are redox sensitive in AngII-stimulated neurons leading to sustained CaMKIIα activation, which drives the inhibition of I_{Kv} and contributes to AngII-induced hypertension. We observed that overexpression of wild-type CaMKIIα in cultured neurons or in the brain SFO potentiated AngII-induced reduction of I_{Kv} and exacerbated the increase in blood pressure mediated by AngII stimulation of the SFO, respectively. In contrast, expression of a mutant CaMKIIα (mutCaMKIIα) in which C280 was mutated to an alanine (C280A) and M281 was mutated to a valine (M281V) failed to exacerbate the AngII-induced inhibition of I_{Kv} and also prevented the excessive AngII-induced increase in blood pressure. Overall, this study implicates that redox-sensitive post-translational modification of CaMKIIα is involved in mediating AngII intra-neuronal signaling and the chronic AngII-mediated neurogenic hypertensive response.

2. Methods

2.1. Neuronal cell culture

Mouse catecholaminergic CATH.a neurons (American Type Culture Collection (ATCC), stock no. CRL–11179) were cultured in RPMI 1640 medium supplemented with 8% normal horse serum (NHS), 4% fetal bovine serum, and 1% penicillin-streptomycin at 37 °C with 5% CO_2 as recommended by ATCC. CATH.a neurons were differentiated for 6–8 days before experimentation by adding *N* [6],*2′-O*-dibutyryladenosine *3′,5′* - cyclic monophosphate sodium salt (1 mM, Sigma-Aldrich, St. Louis, MO) to the culture medium as previously described [8]. It should be noted that CATH.a neurons express both AngII type 1 (AT1) and type 2 (AT2) receptors and have been widely used by various groups including ours to study AngII intra-neuronal signaling [6,8,24].

2.2. Construction and generation of wild-type CaMKIIα adenovirus

Replication-deficient recombinant adenovirus (Ad5-CMV) encoding mouse wild-type calcium/calmodulin-dependent protein kinase IIα (Ad.wtCaMKIIα) was generated. Briefly, CaMKIIα plasmid (Origene) was amplified by conventional Polymerase Chain Reaction (PCR) using the HotStart PCR Master Mix (Qiagen, Venlo, Limburg) and the following primers: forward 5′- GAA TTC ATG GCT ACC ATC ACC TGC ACC C – 3'; reverse 5' – GGA TCC TCA ATG CGG CAG GAC GGA - 3'. The PCR product was then run on a 1% agarose gel, followed by gel extraction utilizing Qiagen gel-extraction kit (Qiagen). The CaMKIIα insert was cloned using a pJet1.2 clone jet kit (Thermo Scientific), transformed into DH5 alpha bacteria and grown overnight onto ampicillin treated plates at 37°c in a bacterial incubator. Bacterial colonies were picked and grown in LB media overnight by shaking. PureLink Quick Plasmid DNA Miniprep Kits (Invitrogen, Grand Island, NY) was used to isolate CaMKII plasmid DNA as per manufacturer's instructions. Following quantification of the DNA using Nanodrop 200 spectrophotometer (Thermo Scientific, Waltham, MA) 1 μg DNA was digested with EcoR1 and BaMH1 restriction enzymes and then run on a 1% agarose gel to confirm the presence of the correct insert. The plasmid samples were then sent off to the UNMC Gene Sequencing Core. Once the CaMKIIα sequence was confirmed it was then cloned into an expression plasmid, transformed into bacteria, DNA isolated, digested using restriction enzymes and again sent to the UNMC Gene sequencing core to confirm the presence of correct CaMKIIα sequence in the expression plasmid. Adenoviral vectors were then constructed, purified and provided by the University of Iowa Gene Vector Core, as previously described [25]. To determine the optimal overexpression of wtCaMKIIα, CATH.a neurons were transduced with Ad.wtCaMKIIα on day 3 of differentiation at 10, 25 and 50 MOI and Western blot analysis was performed to confirm CaMKIIα overexpression. Empty adenoviral vectors (Ad.Empty) or GFP-expressing adenovirus (Ad.GFP) were used as controls.

2.3. Site-directed mutagenesis and mutant CaMKIIα adenovirus generation

Replication-deficient recombinant adenovirus (Ad5-CMV) encoding mouse mutant calcium/calmodulin-dependent protein kinase IIα (Ad.mutCaMKIIα) was generated. Primer sequences were made specifically to mutate cysteine 280 to alanine (C280A) and methionine 281 to valine (M281V) with overlapped extension polymerase chain reaction (PCR). Briefly two sections of the wt-CaMKIIα plasmid (Origene) were amplified by conventional PCR using HotStart PCR Master Mix (Qiagen, Venlo, Limburg) with the following two set of primers: Section 1 forward 5′- GAA TTC ATG GCT ACC ATC ACC TGC ACC C – 3'; reverse 5' – CCA CGG TCT CCT GTC TGT GCA - 3'. Section 2 forward 5′- GTG CAC AGA CAG GAG ACC ACC GTG – 3'; reverse 5' – GGA TCC TCA ATG CGG CAG GAC GGA - 3'. The next set of steps involving cloning using pJet1.2 PCR clone jet kit (Thermo Scientific) and bacterial colony culture was similar to the Ad.wtCaMKIIα generation procedure described above. Adenoviral vectors encoding mutCaMKIIα were constructed, purified and provided by the University of Iowa Gene Vector Core, as previously described [25].

2.4. Western blot analysis

Protein expression of CaMKIIα and actin was determined using standard Western blot analysis in lysates prepared from differentiated CATH.a neurons either non-transduced or transduced (10–50 MOI) with Ad.wtCaMKIIα or Ad.mutCaMKIIα. Briefly, cells were harvested in lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor (Thermo Scientific). Cell lysates (25–30 μg of protein per lane) were loaded onto gels, separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with primary antibodies directed against CaMKIIα (1:1000 dilution, Abcam, Cambridge, UK) and actin (1:1000 dilution, Sigma-Aldrich) at 4 °C overnight. After incubation with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000 dilution, Thermo Scientific, Waltham, MA), bands were visualized using the Pierce

enhanced chemiluminescence detection system (Thermo Scientific, Rockford, IL). Densitometric analysis of band density was determined using NIH ImageJ analysis software. Values were normalized to actin to correct for any variations in protein loading.

2.5. Electrophysiological record of voltage-gated K^+ currents

Differentiated CATH.a neurons either non-transduced or transduced with control adenovirus (Ad.Empty; 25MOI), Ad.wtCaMKIIα (25MOI) or Ad.mutCaMKIIα (25MOI) were used to measure K^+ currents (I_{KV}). I_{KV} was recorded by the whole-cell configuration of the patch-clamp technique using an Axopatch 200-B patch-clamp amplifier (Axon Instruments, Inc, Union City, CA). Briefly, in the voltage-clamp experiments, resistance of the patch pipette was 4–6 MΩ when filled with the following solution (in mM): 130 KCl, 2 $MgCl_2$, 0.25 $CaCl_2$, 5 EGTA, 1 Mg-ATP, 0.1 Tris-GTP, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 8 glucose, pH 7.2. The extracellular solution consisted of (in mM): 137 NaCl, 5.4 KCl, 1.35 $CaCl_2$, 2 $MgCl_2$, 0.3 NaH_2PO_4, 10 HEPES, 10 sucrose, pH 7.4. To selectively record I_{KV}, Na^+ and Ca^{2+} channels were blocked by 0.5 µM tetrodotoxin (TTX, Sigma-Aldrich) and 0.3 mM $CdCl_2$ (Sigma Aldrich) respectively. Current traces were sampled at 10 kHz and filtered at 5 kHz. Holding potential was −80 mV and current-voltage (I–V) relationship was elicited by 10 mV step increments to potentials between −80 and + 80 mV for 400 ms. The change of I_{KV} with AngII was tested by superfusing CATH.a neurons with AngII (100 nM) for 5 min and repeating the voltage pulse regimen. To confirm the observed changes with AngII, I_{Kv} was recorded again after 15 min of washout. Cells responded to AngII stimulation and then recovered with the washout were considered for data analysis. Current density of I_{Kv} was calculated by dividing the respective current by cell membrane capacitance (C_m). The C_m of CATH.a neurons utilized in the study ranged from 10 to 60 pF and was not significantly different between the groups. The pClamp 10.2 program (Axon Instruments) was used for data acquisition and analysis. All experiments were carried out at room temperature.

2.6. Mouse studies

All animal procedures were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Adult C57BL/6 male mice (20–25 g body weight; Harlan, Indianapolis, IN) were used for physiological and immunohistochemical studies. Mice were provided standard chow and water ad libitum. Mice were surgically instrumented with intracerebroventricular (ICV) cannulas for administration of either AngII or adenoviruses to the brain SFO. Mean arterial pressure (MAP), systolic and diastolic blood pressure, and heart rate (HR) were recorded daily with radiotelemetry (Data Sciences International, DSI), as previously described [4,24]. Following 1 week of recovery from surgery, blood pressure and heart rate responses were recorded in conscious, unrestrained mice immediately prior to, during and after acute ICV injection of AngII (350 ng). Then, mice were ICV injected with Ad.GFP (control adenovirus) or Ad.wtCaMKIIα (5×10^7 plaque forming units), and three days later mice were again ICV injected with AngII (350 ng) while blood pressure and heart rate were monitored.

To generate AngII-dependent hypertensive mice, osmotic minipumps (Alzet, Durect) were implanted subcutaneously 3 days after ICV injection of adenovirus (Ad.GFP, Ad.wtCaMKIIα, and Ad.mutCaMKIIα) and delivered AngII (400 ng/kg/min) for approximately 3 weeks [4,24]. Control mice received subcutaneous osmotic minipumps filled with saline.

2.7. Immunohistochemistry

Mice were euthanized with intraperitoneal injection of fatal plus (40 mg/kg, Henry-Schrein, Inc., Melville, NY) after 7 days of ICV adenovirus injection. Brains were removed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) and then stored in 30% sucrose in PB overnight prior to sectioning. Cryostat brains sections (20 µm) containing the SFO were prepared and immunohistochemistry was carried out, as previously described [24]. Briefly, sections were incubated for 1 h with blocking buffer (10% NHS in 0.1 M PB). Tissue sections were then incubated at 4 °C overnight with primary antibody against CaMKIIα (1:200, rabbit monoclonal antibody, Abcam), prepared in 0.1 M PB containing 2% NHS and 0.3% Triton X-100. The sections were then washed in 0.1 M PB and incubated with Alexa Fluor 598 secondary antibody (1:200 dilution, Invitrogen, Carlsbad, CA) at room temperature for 2 h. Fluorescent images were detected by confocal laser scanning microscopy (Zeiss LSM 510 Meta).

2.8. Statistical analysis

All data are presented as means ± standard error of the mean (SEM). Data were analyzed by Student's t-test for two group comparisons or by ANOVA for multiple group comparisons with Newman Kewls post-hoc test. *P*-value less than 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 5.0 statistical and graphical software.

3. Results

3.1. Wild-type CaMKIIα overexpression, but not mutant CaMKIIα, potentiates AngII-mediated inhibition of neuronal K^+ current (I_{Kv}) in CATH.a neurons

To investigate the effect of wtCaMKIIα overexpression on the AngII-dependent changes in neuronal electrophysiological properties, I_{Kv} was measured in non-transduced, Ad.Empty (25 MOI) or Ad.wtCaMKIIα (25 MOI)-transduced CATH.a neurons. Consistent with previous studies [8], acute AngII (100 nM) superfusion for 5 min inhibited I_{Kv} in non-transduced and Ad.Empty-transduced CATH.a neurons, as seen in the representative traces of evoked K^+ currents (Fig. 1A). Interestingly, AngII superfusion caused a further significant reduction in I_{Kv} (Fig. 1A) in Ad.wtCaMKIIα-transduced CATH.a neurons (n = 7) compared to non-transduced (n = 7) or Ad.Empty-treated neurons (n = 7), as shown in the summary data (Fig. 1B). In contrast, expression of mutant CaMKIIα in CATH.a neurons via adenovirus-mediated gene transfer (Ad.-mutCaMKIIα, 25 MOI) failed to potentiate the AngII-induced inhibition of I_{Kv} (Fig. 1B). Western blot analysis confirmed overexpression of wtCaMKIIα or mutant CaMKIIα in neurons transduced with Ad.-wtCaMKIIα or Ad.mutCaMKIIα, respectively, compared to non-transduced and Ad.Empty-treated cells (Fig. S1). These data indicate that overexpression of wtCaMKIIα exacerbates the AngII-induced inhibition of neuronal K^+ channel current, and, more importantly, suggest that cysteine 280 and methionine 281 in CaMKIIα are required to mediate this potentiated AngII response.

3.2. Overexpression of wtCaMKIIα in the brain SFO potentiates the central acute AngII-induced increase in mean arterial pressure (MAP)

To determine the role of wtCaMKIIα overexpression in the brain SFO on the acute, central actions of AngII *in vivo*, C57BL/6 male mice underwent radiotelemetry implantation and ICV cannula surgeries. Following a week's recovery, blood pressure and heart rate responses to ICV administered AngII (350 ng) were recorded in conscious mice. As seen in the representative recordings, mice receiving acute ICV AngII prior to ICV administration of Ad.GFP or Ad.wtCaMKIIα exhibited the characteristic increase in blood pressure as compared to their baseline (Fig. 2A). Interestingly, 3 days post-adenovirus injection, the AngII-mediated rise in blood pressure was potentiated in mice ICV-injected with Ad.wtCaMKIIα compared to Ad.GFP-injected mice. The peak change in MAP following acute ICV AngII injections in Ad.wtCaMKIIα

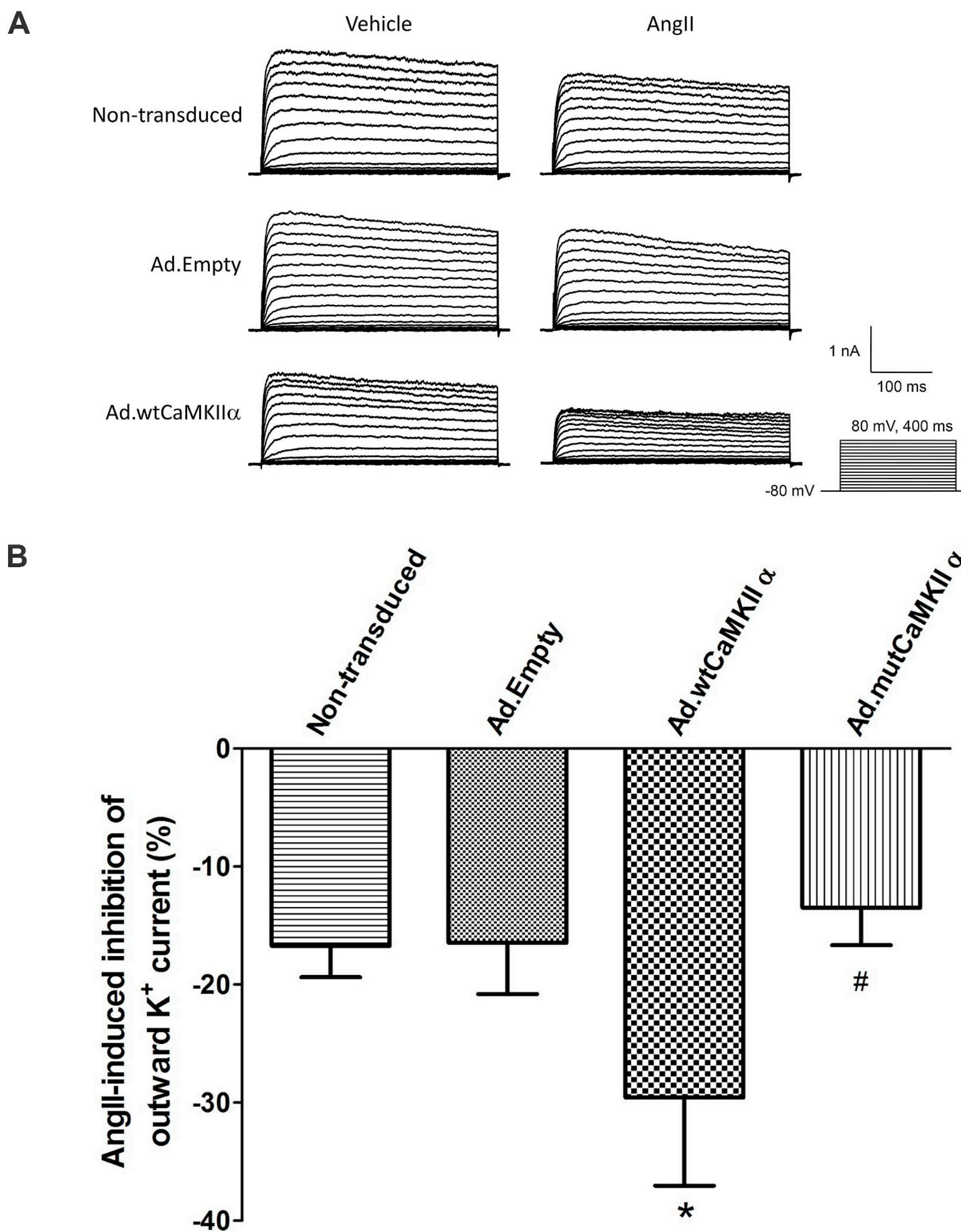

Fig. 1. Wild-type CaMKIIα overexpression, but not mutant CaMKIIα, potentiates the AngII-mediated inhibition of outward K^+ current in CATH.a neurons. A) Representative electrophysiological traces of evoked K^+ currents in non-transduced, Ad.Empty (25MOI) or Ad.wtCaMKIIα (25 MOI)-transduced CATH.a neurons superfused with vehicle or 100 nM AngII. B) Percent inhibition of outward K^+ current following 5 min of AngII (100 nM) superfusion in non-transduced, Ad.Empty (25 MOI), Ad.wtCaMKIIα (25 MOI) or Ad.mutCaMKIIα (25 MOI)-transduced CATH.a neurons. *$p < 0.05$ vs. non-transduced and Ad.Empty. $^{\#}p < 0.05$ vs. Ad.wtCaMKIIα. n = 4–8 cells/group.

(n = 9) and AdGFP (n = 9) mice are summarized in Fig. 2B. Heart rate did not change significantly between the groups (data not shown). In addition, the duration of the AngII-induced pressor response was longer in wtCaMKIIα overexpressing mice as compared to mice with Ad.GFP (Fig. 2C). The duration was calculated from the start of the peak AngII-mediated rise in blood pressure until the time when blood pressures came back to half the maximal response. Together, these data demonstrate that overexpression of wild-type CaMKIIα in the brain SFO exacerbates the central AngII-induced pressor response and suggest that CaMKIIα in the brain is an important player in central AngII-mediated cardiovascular effects.

3.3. Wild-type CaMKIIα overexpression, but not mutant CaMKIIα, in the brain SFO sensitizes mice to an immediate and sustained increase in blood pressure during subcutaneous AngII infusion

We next evaluated the effect of overexpressing wtCaMKIIα or mutCaMKIIα in the brain SFO on the development and progression of AngII-induced hypertension. Similar to the acute AngII studies (Fig. 2), mice were implanted with radiotelemeters for blood pressure monitoring and ICV cannulas for adenovirus injections. Following 1 week of recovery from surgery, Ad.GFP, Ad.wtCaMKIIα or Ad.mutCaMKIIα was administered ICV. Subcutaneous AngII (400 ng/kg/min) or saline osmotic minipumps were implanted 3 days post-adenovirus injection and blood pressure and heart rate were recorded daily in conscious mice for the next 3 weeks. As shown in Fig. 3, MAP, as well as systolic and diastolic blood pressure in Ad.GFP-injected mice (n = 8) increased gradually with AngII infusion with a peak at around day 16. Elevated blood pressures then returned to baseline levels at day 22–23 when the osmotic minipumps emptied. It should be noted that the gradual increase in blood pressure in Ad.GFP-treated mice during subcutaneous infusion of 400 ng/kg/min of AngII is characteristic of the slow-pressor AngII-induced hypertensive model [4,26,27]. Interestingly, in Ad.-wtCaMKIIα-injected mice (n = 9) AngII infusion resulted in a significant increase in blood pressure within 2 days that continued to increase until day 23 when the minipumps emptied and blood pressure returned back to basal levels. In mice expressing mutCaMKIIα in the SFO (n = 6), there was an increase in blood pressure within 2 days of

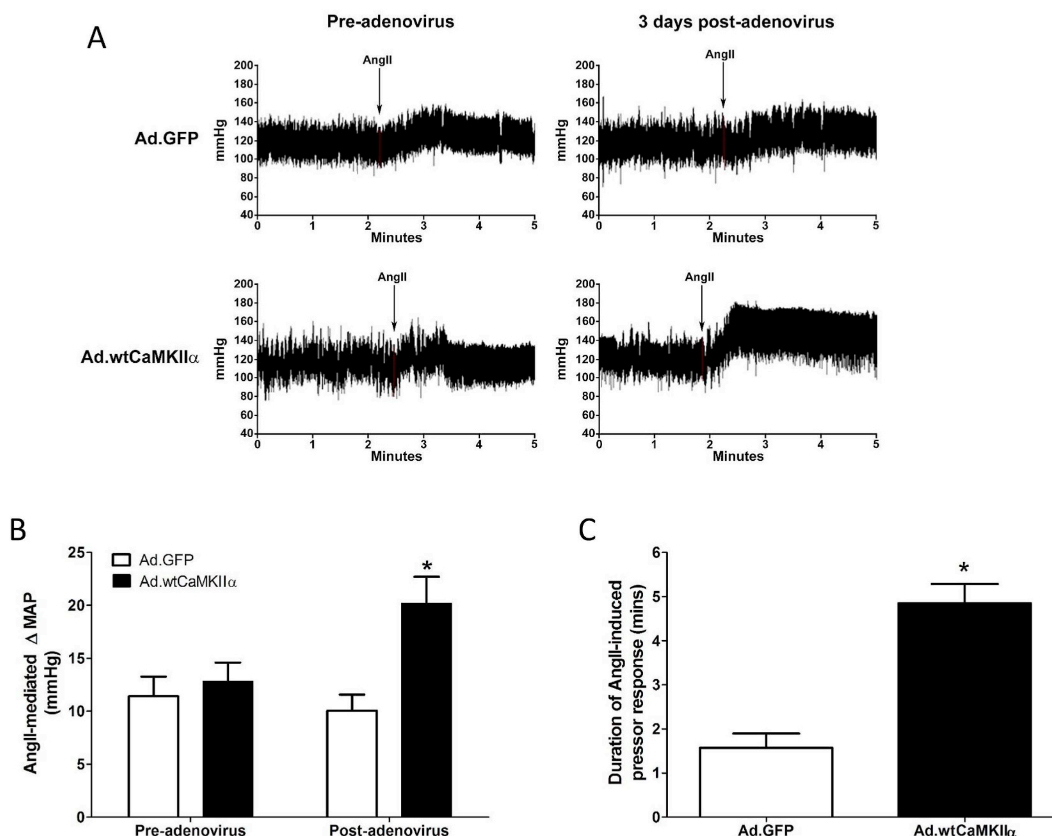

Fig. 2. Overexpression of wtCaMKIIα in the brain SFO potentiates the pressor response of central acute AngII. A) Representative blood pressure tracings and (B) summary data showing AngII-induced acute pressor response in mice ICV injected AngII (350 ng) both pre- and 3 days post-ICV injection of Ad.GFP or Ad.wtCaMKIIα. C) Duration (in minutes) of the AngII-mediated rise in blood pressure in Ad.GFP and Ad.wtCaMKIIα injected mice. *$p < 0.05$ vs. Ad.GFP. n = 8–9 mice/group.

starting AngII, which was similar to the response in Ad.wtCaMKIIα-treated mice. However, blood pressures declined thereafter and continued to rise gradually similar to the Ad.GFP group. The peak increase in MAP (day 15–17 of AngII infusion) was significantly attenuated in the mutCaMKIIα mice (115 mmHg) as compared to the wtCaMKIIα group (133 mmHg). Heart rate was not significantly different among the three groups infused with AngII (data not shown). Blood pressure and heart rate responses did not differ in saline-infused mice ICV

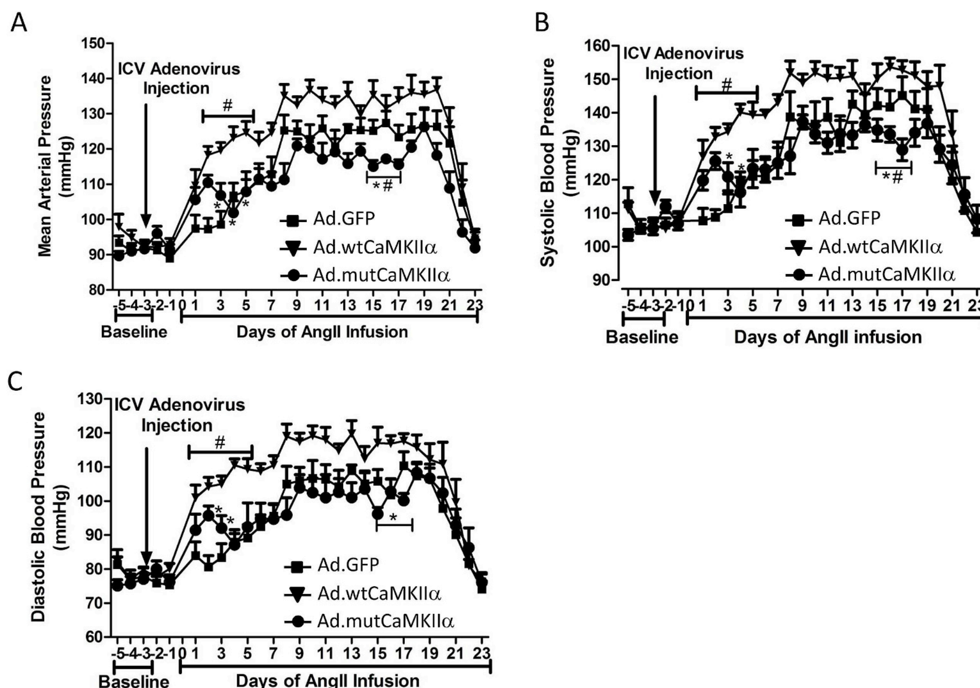

Fig. 3. Wild-type CaMKIIα overexpression, but not mutant CaMKIIα, in the brain SFO sensitizes mice to an immediate and sustained AngII-induced increase in blood pressure. Summary data of (A) mean arterial pressure, (B) systolic blood pressure, and (C) diastolic blood pressure, as measured by radiotelemetry in C57BL/6 male mice at baseline, following ICV injection of Ad.GFP, Ad.wtCaMKIIα or Ad.mutCaMKIIα, and after subcutaneous implantation of osmotic minipumps set to release AngII (400 ng/kg/min). *$p < 0.05$ vs. Ad.wtCaMKIIα. $^{\#}p < 0.05$ vs. Ad.GFP mice. n = 6–9 mice/group.

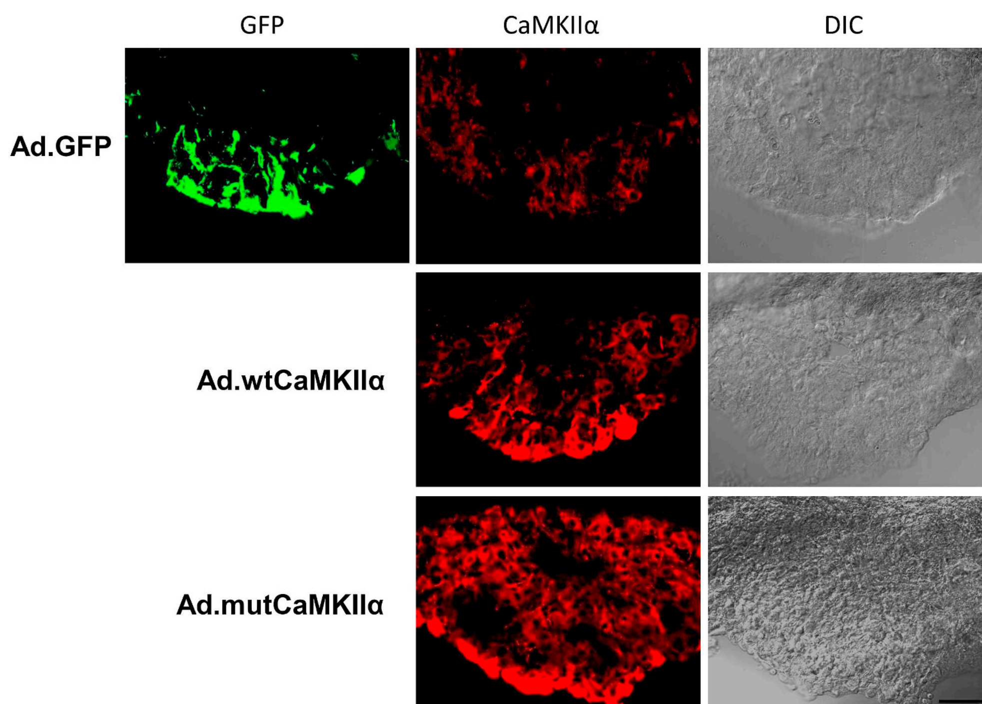

Fig. 4. Adenoviral-mediated over-expression of wtCaMKIIα and mutant CaMKIIα in the brain SFO. Representative confocal microscopy images showing expression of GFP and CaMKIIα in the SFO of mice 1 week after ICV injection of Ad.GFP, Ad.wtCaMKIIα or Ad.mutCaMKIIα. Coronal brain sections (20 µm) were stained for GFP (green fluorescence), and CaMKIIα (red fluorescence). Differential interference contrast (DIC) images show entire SFO tissue. Scale bar = 100 µm.

injected with Ad.GFP, Ad.wtCaMKIIα or Ad.mutCaMKIIα (data not shown).

Importantly, to confirm efficient adenovirus-mediated overexpression of wtCaMKIIα and mutCaMKIIα in the SFO following ICV administration of Ad.wtCaMKIIα or Ad.mutCaMKIIα, respectively, immunohistochemistry was performed on brain tissue sections 7 days post ICV adenovirus injections. Control mice received ICV Ad.GFP. As shown in the representative confocal microscopy images (Fig. 4), the SFO from AdwtCaMKIIα- and Ad.mutCaMKIIα-injected mouse brains exhibited higher levels of CaMKIIα protein levels in comparison to the SFO from Ad.GFP-treated mice. Overall, these studies indicate that wtCaMKIIα overexpression in the brain SFO sensitizes mice to an immediate increase in blood pressure at an initial subpressor dose of subcutaneous AngII. Furthermore, mutation of redox-sensitive cysteine and methionine residues in CaMKIIα prevents the sensitization mechanism and attenuates the peak AngII-induced rise in blood pressure.

4. Discussion

Dysregulation in AngII intra-neuronal signaling mechanisms contributes to the pathogenesis of neuro-cardiovascular diseases, such as hypertension and heart failure [1,2]. In central neurons, AngII activates redox mechanisms including NADPH oxidases and increases generation of reactive oxygen species, particularly superoxide [5]. Many studies have clearly demonstrated that elevated levels of reactive oxygen species in neurons located in cardiovascular control brain regions mediate enhanced neuronal activation and drive sympathoexcitation, which are known to increase systemic blood pressure [3–5,23]. However, the underlying mechanism(s) by which an increase in neuronal reactive oxygen species following AngII stimulation leads to neuronal firing, sympathoexcitation, and elevated in blood pressure remains unclear. Herein, we report that adenoviral-mediated overexpression of wild-type CaMKIIα (Ad.wtCaMKIIα), the isoform of CaMKII highly expressed in neurons, in central neurons potentiates the AngII-induced reduction in neuronal K^+ current and AngII-induced hypertension. In addition, mutation of specific cysteine (C280) and methionine (M281) residues in CaMKIIα attenuates the potentiated AngII response observed with wtCaMKIIα overexpression. The main findings of this study can be summarized as follows: (1) the potentiated AngII-mediated inhibition of neuronal K^+ current (I_{KV}) following wtCaMKIIα overexpression in CATH.a neurons is absent in mutant CaMKIIα-transduced neurons; (2) overexpression of wtCaMKIIα in the brain SFO potentiates the central acute AngII-induced pressor response; (3) wtCaMKIIα overexpression in the SFO sensitizes mice to an immediate increase in blood pressure at an initial subpressor dose of subcutaneous chronic AngII infusion; and (4) mutant CaMKIIα in the SFO prevents the sensitization mechanism and attenuates the peak AngII-induced rise in blood pressure.

Previous studies have identified signaling proteins, such as CaMKIIα and PKC, as downstream mediators of AngII signaling in central neurons [6,7,9]. These studies have shown a clear contribution of CaMKIIα in mediating the AngII-induced inhibition of neuronal ion current, specifically K^+ current, and the subsequent increase in neuronal firing both in primary neuronal cultures as well as in neuronal cell lines. Previous results from our lab suggest redox-dependent activation of CaMKIIα, as measured by increased CaMKIIα phosphorylation in AngII-stimulated CATH.a neurons that could be blocked by overexpressing superoxide dismutase [8]. Additionally, Erickson et al. have demonstrated that AngII can induce oxidation of cardiac CaMKII (CaMKIIδ) at paired methionine residue (M281/282) and sustain CaMKIIδ activity independent of Ca^{2+}/CaM binding [12]. This sustained activity of CaMKIIδ plays an important role in AngII-mediated apoptosis in ischemic heart and under myocardial infarction conditions [12–14]. Further studies have also elucidated that CaMKIIα can undergo S-nitrosylation at cysteine 280 and 289, and mutation of either cysteine residue attenuates nitric oxide-induced excitotoxic neuronal cell death [15]. Although these studies indicate the importance of reactive oxygen species in CaMKII activation, the role of redox sensitive CaMKIIα in AngII-dependent intra-neuronal signaling and AngII-mediated hypertension is unknown.

To determine the role of redox-dependent activation CaMKIIα in mediating AngII-induced reduction in neuronal I_{Kv}, we used adenoviral-mediated overexpression of wtCaMKIIα in CATH.a neurons and measured changes in outward K^+ current. AngII superfusion inhibited I_{Kv} by almost 16% both in non-transduced as well as in Ad.Empty-transduced neurons. Interestingly, this effect was significantly exaggerated in Ad.wtCaMKIIα-transduced neurons (37% inhibition). In contrast, CATH.a neurons that were transduced with mutCaMKIIα adenovirus had a 13% inhibition in outward K^+ current, which was similar to

control neurons. These results strongly suggest that cysteine 280 and methionine 281 are required for wild-type CaMKIIα to potentiate the AngII-induced inhibition of outward I_{Kv}. Further studies are needed to determine if overexpression of wtCaMKIIα in neurons results in changes in protein levels and/or membrane localization of K^+ channels, or if the potentiated AngII response is a result of wild-type CaMKIIα directly acting on K^+ channels to regulate their activation.

Brain AngII is known as a potent regulator of arterial blood pressure and when administered acutely via ICV it elicits a well-characterized increase in blood pressure [16–19]. As seen in the representative blood pressure tracings in Fig. 2, acute ICV injection of AngII caused a rapid and relatively short-lived increase in mean arterial pressure in Ad.GFP-treated mice. This AngII response was significantly potentiated in mice overexpressing wtCaMKIIα in the SFO. We did not observe changes in baseline blood pressure with wtCaMKIIα overexpression. We next wanted to evaluate the role of CaMKIIα in the SFO on the development and progression of AngII-dependent hypertension. In order to do these studies, we utilized the slow-pressor chronic AngII-induced hypertensive model that has been extensively used by our lab and others to investigate intra-neuronal signaling mechanisms in AngII-mediated hypertension [4,24,26,27]. Intriguingly, we observed a significant rise in blood pressure in Ad.wtCaMKIIα-injected mice immediately (i.e. within 2 days) after the start of AngII infusion as compared to a more gradual rise in Ad.GFP-injected mice. Further, this robust and immediate increase in blood pressure was sustained throughout AngII infusion and resulted in the peak rise in blood pressure being significantly higher in Ad.wtCaMKIIα injected mice as compared to Ad.GFP group (133 mmHg in Ad.wtCaMKIIα vs. 125 mmHg in AdGFP group at days 15–17). We conclude that mice overexpressing wtCaMKIIα in the brain SFO are sensitized to the initial subpressor dose of AngII infusion used in our study (i.e. 400 ng/kg/min). Although further studies are needed to determine the precise mechanism of action by which wtCaMKIIα overexpression in the SFO sensitize mice to peripherally infused AngII, we predict the elevated levels of wtCaMKII enhance AngII-dependent intra-neuronal signaling in neurons residing in the blood-brain barrier deficient SFO. Further, we speculate that this enhanced signaling results in activation of SFO neurons, perhaps via potentiation of outward K^+ current, projecting to cardiovascular control regions behind the blood-brain barrier, such as the paraventricular nucleus (PVN), which are known to drive sympathoexcitation and increase blood pressure.

Lastly, in our current study, we observed that mice expressing mutant CaMKIIα in the SFO had a similar rise in blood pressure within 2 days of AngII infusion as observed in the wtCaMKIIα group. However, blood pressure declined after 2 days and followed a more gradual increase, similar to the Ad.GFP-injected mice infused with AngII. The peak AngII-induced increase in blood pressure in mice with mutCaMKIIα was also significantly lower (115 mmHg). We speculate that the initial rise in blood pressure in mutCaMKIIα is due to a similar sensitization mechanism as seen in wtCaMKIIα mice. However, considering cysteine 280 and methionine 281 are mutated and previous studies suggest the redox modulation of CaMKII results in sustained activity even in the absence of Ca^{2+}/calmodulin [12,14], we predict this sensitization is lost as the mutant CaMKIIα is unable to sustain its activity which results in the subsequent gradual increase in blood pressure similar to Ad.GFP mice.

Although the current study provides new insight into AngII intra-neuronal redox signaling mechanisms and indicates that C280 and M281 of neuronal CaMKIIα are redox-sensitive targets of AngII, there are various limitations of this study that must be addressed. First, considering our mutant CaMKIIα included mutation of C280 and M281, it remains unknown if one or both of these redox-sensitive amino acids contribute to the actions of CaMKIIα in AngII stimulated neurons. Second, numerous previous studies from various groups, including ours [3–5,8,24], have identified superoxide as the primary ROS elevated in AngII-stimulated neurons. However, it is unlikely that the thiol in cysteine and methionine is sensitive to oxidation by superoxide. As such, it remains unclear precisely how C280 and/or M281 in CaMKIIα are oxidized in AngII activated neurons. Additional studies are needed to determine if hydrogen peroxide or some other ROS are involved in oxidizing C280 and/or M281. Lastly, future studies involving mass spectrometry and proteomic approaches are needed to definitively describe the oxidative post-translational modifications of CaMKIIα in AngII-stimulated neurons.

In summary, the experimental data presented herein indicate that neuronal CaMKIIα is an important redox-sensitive signaling protein contributing to AngII intra-neuronal signaling and hypertension. More specifically, we showed that wtCaMKIIα overexpression exacerbates the AngII-mediated inhibition of neuronal K^+ current, which was blocked with mutation of redox-sensitive residues (i.e. C280 and M281) in CaMKIIα. In addition, wtCaMKIIα overexpression in the SFO potentiated the central acute AngII-mediated pressor response, and sensitized mice to an immediate increase in blood pressure at an initial subpressor dose of subcutaneous chronic AngII. Importantly, mice expressing mutCaMKIIα in the SFO were protected from the sensitization mechanism and also had a significant lower peak AngII response. In conclusion, these studies may help to identify potential redox-sensitive therapeutic targets, such as CaMKIIα, for the improved treatment of AngII-dependent hypertension.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101230.

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