

An angiotensin II- and NF- κ B-dependent mechanism increases connexin 43 in murine arteries targeted by renin-dependent hypertension

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Aims Connexins (Cx) play a role in the contractility of the aorta wall. We investigated how connexins of the endothelial cells (ECs; Cx37, Cx40) and smooth muscle cells (SMCs; Cx43, Cx45) of the aorta change during renin-dependent and -independent hypertension.

Methods and results We subjected both wild-type (WT) mice and mice lacking Cx40 (Cx40^{-/-}), to either a two-kidney, one-clip procedure or to N-nitro-L-arginine-methyl-ester treatment, which induce renin-dependent and -independent hypertension, respectively. All hypertensive mice featured a thickened aortic wall, increased levels of Cx37 and Cx45 in SMC, and of Cx40 in EC (except in Cx40^{-/-} mice). Cx43 was up-regulated, with no effect on its S368 phosphorylation, only in the SMCs of renin-dependent models of hypertension. Blockade of the renin-angiotensin system of Cx40^{-/-} mice normalized blood pressure and prevented both aortic thickening and Cx alterations. *Ex vivo* exposure of WT aortas, carotids, and mesenteric arteries to physiologically relevant levels of angiotensin II (AngII) increased the levels of Cx43, but not of other Cx. In the aortic SMC line of A7r5 cells, AngII activated kinase-dependent pathways and induced binding of the nuclear factor-kappa B (NF- κ B) to the Cx43 gene promoter, increasing Cx43 expression.

Conclusion In both large and small arteries, hypertension differently regulates Cx expression in SMC and EC layers. Cx43 is selectively increased in renin-dependent hypertension via an AngII activation of the extracellular signal-regulated kinase and NF- κ B pathways.

Keywords Gap junctions • Cx37 • Cx40 • Cx43 • Cx45 • Aorta • Arteries • Angiotensin II • Renin • NF- κ B • Transgenic mice • Hypertension

1. Introduction

The connexin (Cx) channels of gap junctions provide pathways for the communication of both endothelial cells (ECs) and smooth muscle cells (SMCs) along the vascular system,¹ in patterns which vary in different species and vessels.^{2,3} Gap junctions allow for spreading of signals coordinating both vasoconstriction and vasodilation. ECs predominantly express Cx37, Cx40, and some Cx43, whereas SMCs are coupled by Cx43 and Cx45, sometimes with minimal levels of Cx40 and Cx37.^{1,4} Conditions leading to alterations in the arterial wall and/or associated with altered mechanical stress of ECs and SMCs induce changes of several Cx^{1,5,6} and, conversely, mice lacking Cx40 or Cx43 feature abnormal regulation of blood pressure.^{5,7–11}

Chronic hypertension results in hypertrophic remodelling of the aortic and carotid walls in humans and animals,^{12–14} as an adaptation to altered hemodynamic.¹⁵ These alterations, which result in increased isobaric stiffness¹⁶ predominantly in the aorta and proximal elastic arteries¹⁵ and in cardiac after-load,¹⁷ also modify vascular Cx. However, it is still unclear whether different Cxs similarly change in various forms of hypertension. We have investigated these questions by comparing mice lacking Cx40, a genetic, renin-dependent model of hypertension,^{7,8,11} with wild-type (WT) mice. Both types of mice were induced to develop a renin-dependent or -independent hypertension by clipping one renal artery (two-kidney, one-clip model; 2K1C)⁵ and by inhibiting nitric oxide

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synthase (*N*-nitro-L-arginine methyl ester model; L-NAME),^{5,7,10,18} respectively.

Our data show that chronic hypertension specifically regulates Cxs in distinct layers of large and small size arteries, by mechanisms that vary for different Cxs. Chronic hypertension up-regulates Cx40 in ECs, and Cx37 and Cx45 in SMCs. In contrast, Cx43 is selectively up-regulated in the SMCs of the renin-dependent models of hypertension, by a hitherto unknown mechanism, which we now demonstrate involves the AngII activation of the ERK and NF- κ B pathways.

2. Methods

2.1 Animal experiments

The investigation confirms with the Guide for the Care and Use of Laboratory Animals published by US National Institution of Health (NIH Publication No. 85-23, revised 1996), mouse care, surgery, and euthanasia procedures were approved by our institution and the Veterinary Office (Lausanne, Switzerland), and confirms with the guide for the care and use of laboratory animals (University of Lausanne, A5552-01). The generation of Cx40^{-/-} mice and their genotypic characterization have been described.^{7,11,19} Breeding pairs were crossed with control C57BL6 partners. Identification of WT and homozygous Cx40^{-/-} mice was performed by PCR of genomic DNA.^{7,11} Three- to 4-month-old animals, from litters obtained after at least 10 backcrosses into the C57/BL6 background, were used in all the experiments. WT and Cx40^{-/-} mice (5–17 mice per group) with a comparable genetic background were compared in each of the following protocols: (i) for the 2K1C model, the left renal artery was clipped with a 0.12 mm internal diameter U-shaped silver clip for 4 weeks;^{5,7,11} (ii) addition to drinking water of either 3 mg/kg/day candesartan (Cilexetil[®], Astra Zeneca) or 10 mg/kg/day ramipril (Aventis),⁷ addition to drinking water of 80 mg/kg/day L-NAME (Sigma), for 3 weeks.

2.2 Measurement of blood pressure

One day before sacrifice, the right carotid artery was penetrated with a catheter connected to a pressure transducer and a computerized data acquisition system,^{5,7,11,18,20} which recorded heart rate and both diastolic and systolic intra-arterial pressure in conscious mice, every 20 s. The system comprised a pressure transducer 156 PC PK 87802 (Honeywell Micro-Switch, Richardson, TX, USA), an HP9000-300 computer (Hewlett-Packard, Palo Alto, CA, USA), and a PP3852A scanner (Hewlett-Packard).²¹ All data are shown as the mean of the diastolic and systolic values (MBP). In some experiments, MBP was monitored in 7-day pre-trained mice by a non-invasive computerized tail cuff method (BP-2000, Visitech Systems). The system automatically performs a cuff inflation and deflation cycle and records pulse rate and blood pressure.^{5,7,11} Records were provided for an average evaluation of blood pressure over a 10-day period. In awake mice, these measurements correlated well with those made by the intra-arterial method (data not shown).

2.3 Measurement of plasma renin

Plasma renin activity (PRA) was measured by a microassay based on angiotensin I (AngI) trapping by an antibody.^{5,7} The amounts of AngI produced during the incubation period were then determined using a sensitive radioimmunoassay with commercially available ¹²⁵I-angiotensin I (Anawa Trading SA).^{5,7}

2.4 Measurement of aorta thickness

Euthanized mice were perfused with PBS through the left ventricle and the aortas were excised for cryosectioning. The combined thickness (intima and media) and the internal diameter of the aorta were measured.²²

2.5 Ex vivo studies

Fragments of freshly excised aortas, carotids, and superior mesenteric arteries were opened longitudinally in DMEM and exposed to various concentrations of angiotensin II (AngII; Calbiochem), in the presence or absence of either 20 μ M candesartan or 20 μ M PD98059 (Calbiochem) for the durations given below.

2.6 Cells

A7r5 cells, a line derived from rat embryonic aorta SMCs,²³ were obtained from Urs Ruegg (University of Geneva and Lausanne, Switzerland)^{24,25} and cultured in Dulbecco's medium. The clone we used in this study expressed the AngII receptor AT1_a (see Supplementary material online, Figure S1).

2.7 Connexin analysis

RNA was analysed by real-time qPCR,^{5,7,11} using specific primers (see Supplementary material online, Table S1). For *en face* immunostaining, aortas were fixed, opened longitudinally, and labelled for Cx40, Cx37,^{2,26} and Cx43, using antibodies AB1726 (Chemicon) and AB1738 (Chemicon), diluted 1:50, respectively. For immunolabelling of sections and western blots, the freshly excised vessels were processed as reported.^{5,27} To prepare enriched ECs or SMCs, aortas were longitudinally opened in 100 μ L PBS, pinned on silicone, and ECs were scraped with a scalpel. The remaining tissue provided enriched SMCs. Samples were homogenized in SDS lysis buffer and run on a 12.5% gel. Membranes were incubated overnight with the antibodies listed above, or antibodies against Cx45 (AB1748, Chemicon), α -tubulin (T5168, Sigma), or β -actin (A5441, Sigma), p-ERK1/2 and ERK1/2 (9101 and 4695, Cell Signaling). To detect phosphorylated Cx43, we used antibodies AB3841 (Chemicon) and sc-22267-r (Santa Cruz), directed against the Ser368 and Ser262 forms, respectively. Immunolabelled proteins were detected by chemiluminescence, and Cx signals normalized to either tubulin or β -actin levels (given that we could not detect tubulin in purified ECs).¹¹

2.8 Mechanism of Cx43 up-regulation

A7r5 cells were transiently transfected using TRANSFAST (Catalys).²⁸ Reporter plasmids consisted of the luciferase gene fused downstream of 699 or 258 bp fragments of the promoter of the rat *Gja1* gene, both of which included a κ B-binding site. The following day, cells were incubated for 4 h in the presence or absence of 2 μ M AngII, and extracts were prepared for dual-luciferase reporter assay (Promega).²⁸ A mutation of the putative nuclear factor-kappa B (NF- κ B)-binding site was generated in the Cx43 promoter by Quick Site-directed mutagenesis (Stratagene, Europe). The PCR amplification was performed using pGL3-258 and pGL3-699 as templates, and the following forward and reverse primers: 5'-TAGTAGGGC TTGTTTCAGtatATGACCAGGGGGGAGAGAAG-3' (NF- κ B sequence underlined and mutagenized nucleotides in lowercase), and 5'-CTTC TCCCTCCCCCTGGTCATATACTGAAACAAGCCCTACTA-3'.

A7r5 cells were cross-linked and processed for chromatin immunoprecipitation.^{28,29} Lysates were immunoprecipitated overnight with antibodies against either NF- κ B p65 (sc-372 X; Santa Cruz Biotechnology) or NF- κ B p50 (sc-1190 X; Santa Cruz). Complexes were eluted, purified, and DNA PCR-amplified to give a 246 bp fragment containing the κ B site of the rat *Gja1* promoter. For ERK and I κ B α western blots, A7r5 cells were processed as described above. To detect NF- κ B, nuclear extracts were prepared using an NE-PER kit (Pierce). The following antibodies were used: p-I κ B α and I κ B α (SC-7977 and SC-203, Santa Cruz), and p-ERK1/2 and ERK1/2 (9101 and 4695, Cell Signaling).

2.9 Statistical analysis

All western blot and qPCR experiments were quantitatively analysed and results are shown as mean + SEM. One-way analysis of variance (ANOVA) was performed to compare the mean values between

groups, using the *post hoc* Bonferroni test, as provided by The Statistical Package for the Social Science (SPSS 17.0, Chicago, IL, USA).

3. Results

3.1 Mouse characteristics

Compared with WT, Cx40^{-/-} mice featured the changes previously reported,^{7,11} including increased blood pressure due to elevated circulating renin (PRA), and a thickening of the aortic media (see Supplementary material online, Table S2). Treatment with candesartan, an antagonist of the AT1 AngII receptor, or ramipril, an inhibitor of the AngII-converting enzyme, did not alter the blood pressure or the aorta thickness of WT mice, in spite of a significant increase in PRA. Both treatments reduced to control the values of blood pressure and aorta thickness of Cx40^{-/-} mice, without altering PRA (see Supplementary material online, Table S2).

The 2K1C treatment increased the blood pressure, PRA, and aorta thickness of WT mice to the levels observed in Cx40^{-/-} mice, which were not affected by this protocol (see Supplementary material online, Table S2). The administration of L-NAME increased blood pressure by 19% and aorta thickness, but not PRA of WT mice. A smaller (8%) increase in blood pressure was observed in Cx40^{-/-} mice, whose PRA remained significantly higher than that in WT littermates (see Supplementary material online, Table S2). The expression of the AT1_a receptor was lower in aortas of Cx40^{-/-} mice than in those of WT littermates, and, in the latter animals, was increased by the candesartan and ramipril treatments. In WT mice, the 2K1C procedure decreased the levels of the AT1_a receptor to the levels of Cx40^{-/-} animals, which were not significantly affected by this procedure. The L-NAME treatment did not alter the expression of the AT1_a receptor (see Supplementary material online, Table S2).

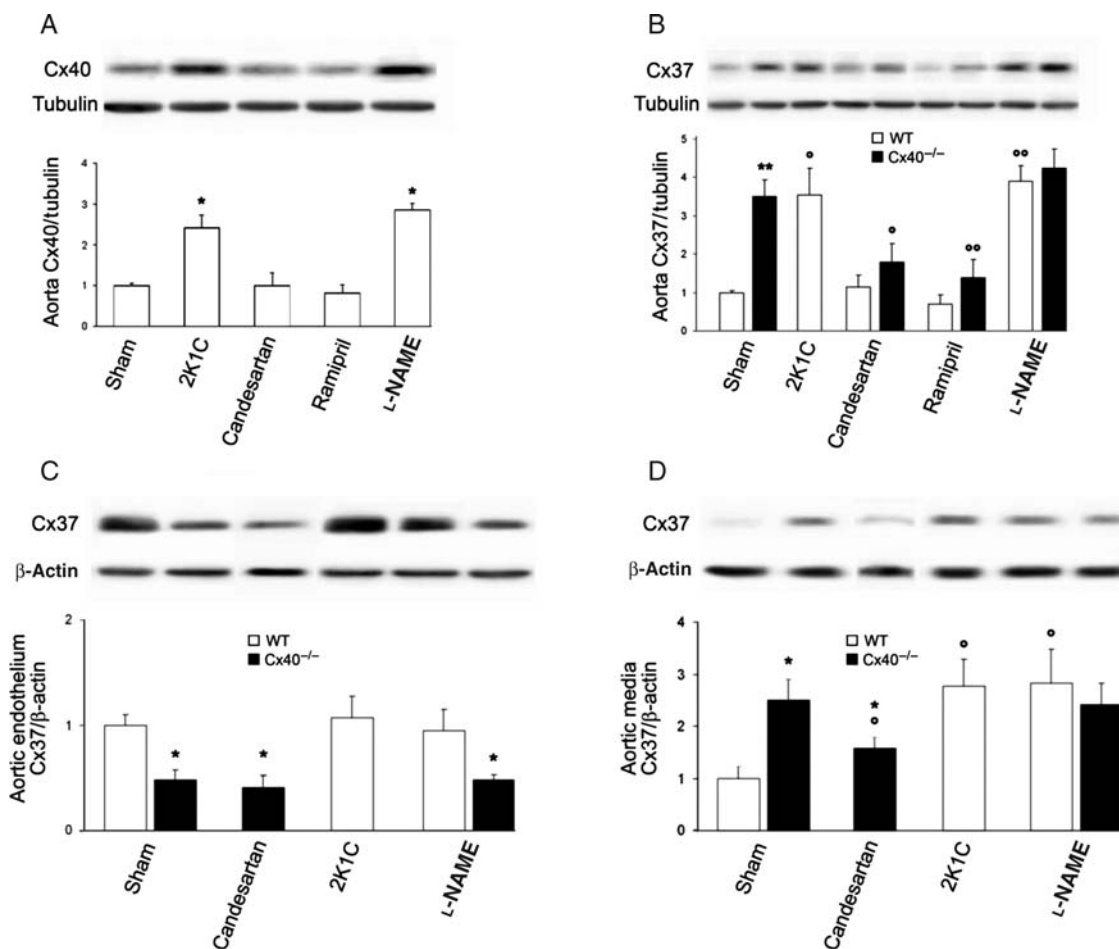


Figure 1 Cx40 and Cx37 increase in the aorta of hypertensive mice. (A) Western blots showed increased Cx40 in renin-dependent and -independent hypertensive WT mice. (B) Cx37 was also higher in the aortas of hypertensive animals. Treatment with candesartan or ramipril reduced the expression of Cx37 in the spontaneously hypertensive Cx40^{-/-} mice. (C) Western blots demonstrated that Cx37 levels did not change in the aortic intima of either 2K1C or L-NAME hypertensive mice, and were lower in Cx40^{-/-} than in WT animals. Treatment with candesartan did not affect Cx37 levels in the endothelium of Cx40^{-/-} mice. (D) Cx37 was less abundant in the SMCs than in the ECs of WT controls, whereas it increased in the SMCs of Cx40^{-/-} mice. A Cx37 increase occurred in the media of all hypertensive mice. The levels of SMC Cx37 were also altered after candesartan treatment in Cx40^{-/-} mice. Data represent mean + SEM of four experiments. **P* < 0.05, ***P* < 0.01 vs. the respective WT animals; °*P* < 0.05, °°*P* < 0.01 vs. WT or Cx40^{-/-} untreated or sham-operated animals. (E) *En face* immunostaining showed that Cx40, but not Cx37, was increased between ECs of hypertensive mice. (F) Sections confirmed that Cx40 was solely expressed in ECs, whereas Cx37 was detectable in ECs and in the SMCs of hypertensive mice. L, lumen; M, media. Bar represents 30 μm.

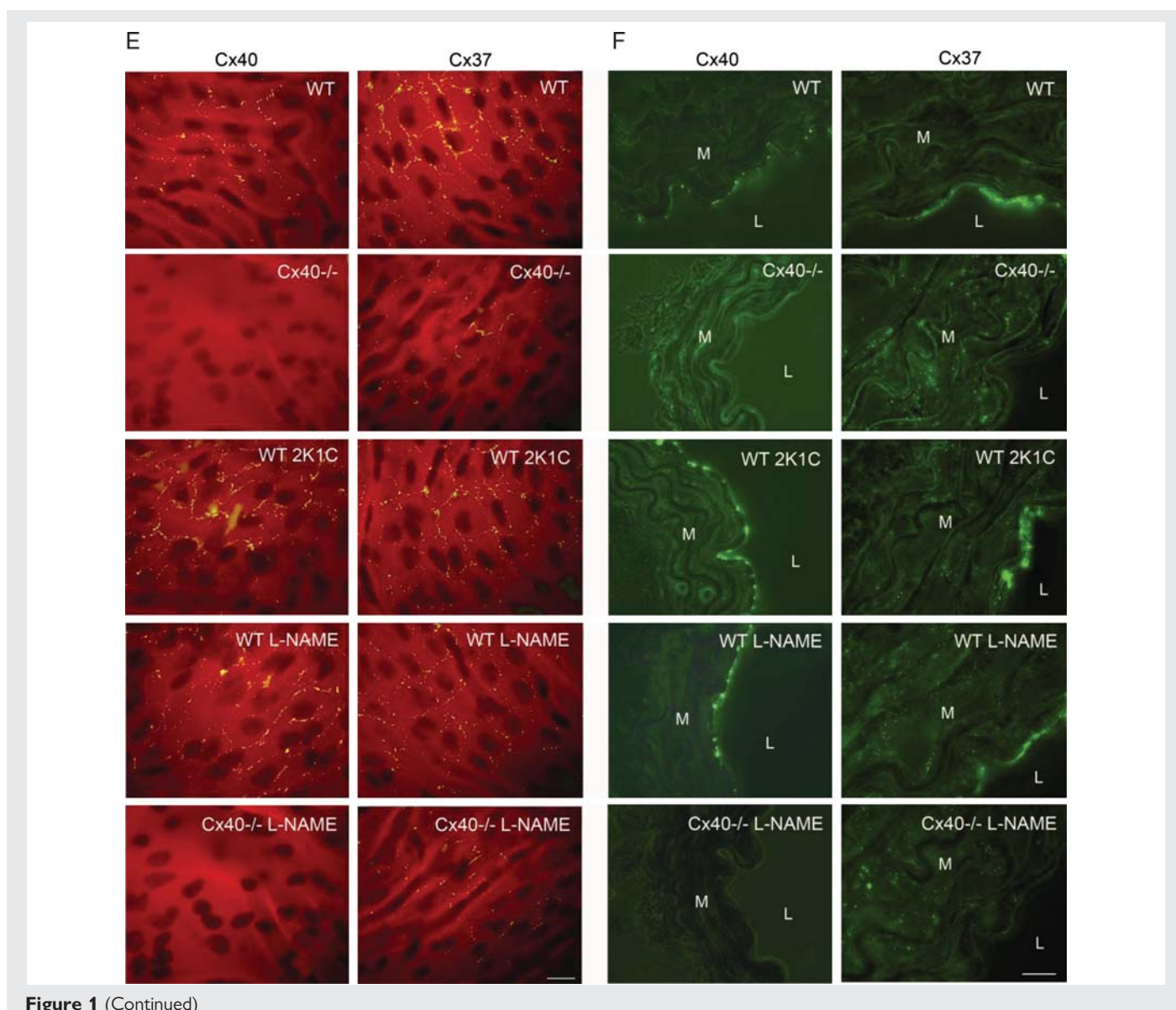


Figure 1 (Continued)

3.2 Multiple connexins increase in the aorta of hypertensive mice

After 4 weeks of hypertension, Cx40 increased in WT mice submitted to either the 2K1C or L-NAME treatment (Figure 1A), due to a transcriptional up-regulation (see Supplementary material online, Figure S2A). Immunostaining showed that Cx40 was increased solely between ECs (Figure 1E and F, left panels). At the same time point, Cx37 was also increased in the aortas of all WT mice made hypertensive, as well as in hypertensive Cx40^{-/-} mice (Figure 1B and see Supplementary material online, Figure S2B), due to an up-regulation in SMCs (Figure 1C and D). Accordingly, immunostaining and western blot revealed no significant change of Cx37 in ECs, except for the expected² decrease in Cx40^{-/-} mice (Figure 1C, E and F, right panels). Treatment with either candesartan or ramipril reduced Cx37 expression in Cx40^{-/-}, but not WT mice (Figure 1B and see Supplementary material online, Figure S2B). The SMC, but not the EC, distribution was altered after both the candesartan (Figure 1C and D) and the ramipril treatments (data not shown). Cx45 was also increased in the aortic SMCs of all hypertensive mice

(Figure 2A) and was reduced under treatment with either candesartan or ramipril in Cx40^{-/-} but not WT mice (Figure 2A, left panel, and see Supplementary material online, Figure S2C).

Cx43 increased only in renin-dependent hypertension (Figure 2B), due to a transcriptional up-regulation (see Supplementary material online, Figure S2D) which, as judged by its blockade under candesartan or ramipril treatment, required activation of the renin-angiotensin system (RAS) (Figure 2B and see Supplementary material online, Figure S2D). Immunostaining showed that most of Cx43 was expressed by aortic SMCs (Figure 2C). Antibodies to Ser262 and Ser368 showed that these changes did not affect the proportion of phosphorylated Cx43 (see Supplementary material online, Figure S3). These results show that chronic hypertension is associated with an up-regulation of all major aortic Cxs, which are differentially regulated by renin-dependent and -independent mechanisms.

3.3 Cx43 is increased by AngII

Given that Cx43 was the only Cx which selectively increased in renin-dependent hypertension, and that AngII is the main RAS

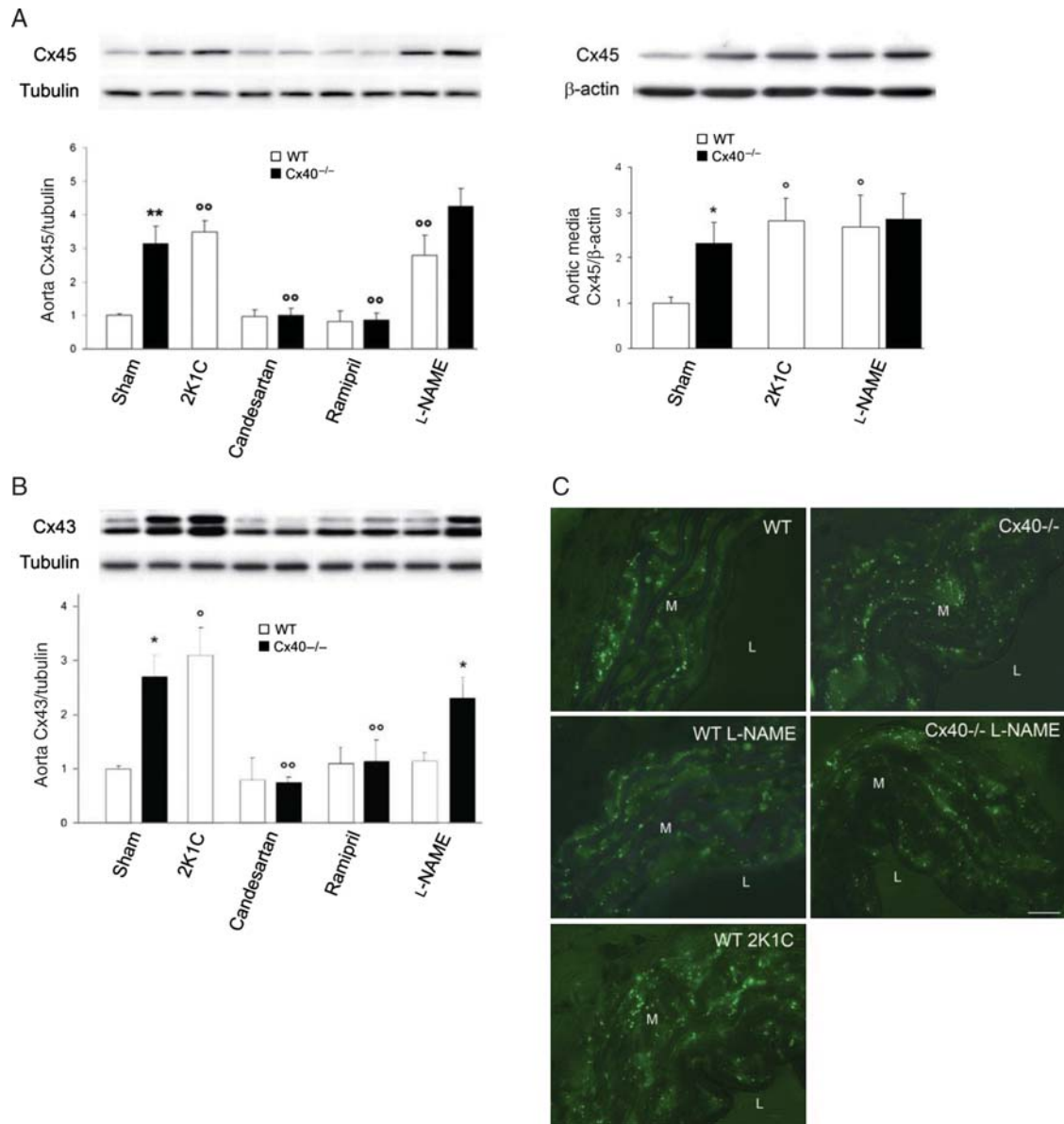


Figure 2 Cx43 and Cx45 are differently affected by renin-dependent and -independent hypertension. (A) Cx45 increased in all WT mice made hypertensive, and this increase was blocked in Cx40^{-/-} mice by RAS inhibitors (left panel). Cx45 was specifically increased in the aortic SMCs of hypertensive mice (right panel). (B) In contrast, Cx43 increased only in aortas of renin-dependent models (Cx40^{-/-} and 2K1C). Data represent mean + SEM of four experiments. * $P < 0.05$, ** $P < 0.01$ vs. the respective WT animals; ^o $P < 0.05$, ^{oo} $P < 0.01$ vs. WT or Cx40^{-/-} untreated or sham-operated animals. (C) Immunostaining showed Cx43 throughout the media of all aortas. Increased numbers of immunospots were observed in the media of all Cx40^{-/-} and hypertensive 2K1C WT mice. M, media; L, lumen. Bar represents 30 μ m.

activator in this form of hypertension,^{30,31} we investigated the relationship between these two peptides. By exposing freshly isolated aortas of WT mice to AngII, we observed that within 4 h, 10 nM AngII, a concentration that mimics the physiological levels of the vasoconstricting peptide,^{18,32–35} significantly increased the levels of Cx43. However, the two- to three-fold increase in this protein which was observed *in vivo* was only reached with micromolar concentrations (Figures 3A and B). Therefore, we selected a concentration of 2 μ M AngII for all other experiments. Western blots showed that within 2 h, 2 μ M AngII increased Cx43 in WT SMCs (Figure 3C), and the phosphorylation of ERK1/2 (Figure 3D).

This phosphorylation was not increased following the L-NAME treatment (Figure 3E), which also did not alter Cx43 expression (Figure 2B). In the aortas of Cx40^{-/-} mice, AngII induced a smaller and delayed increase in Cx43, and prevented the down-regulation of Cx43 expression which occurred in the absence of the hormone (Figure 3F). Under this condition, the levels of Cx43 decreased in the aorta of Cx40^{-/-} mice to reach control values within 48 h. (Figure 3F), due to rundown of AngII, which is natively high in these animals.^{7,8} These results show that Cx43 is up-regulated in parallel with the ERK pathway, due to RAS activation.

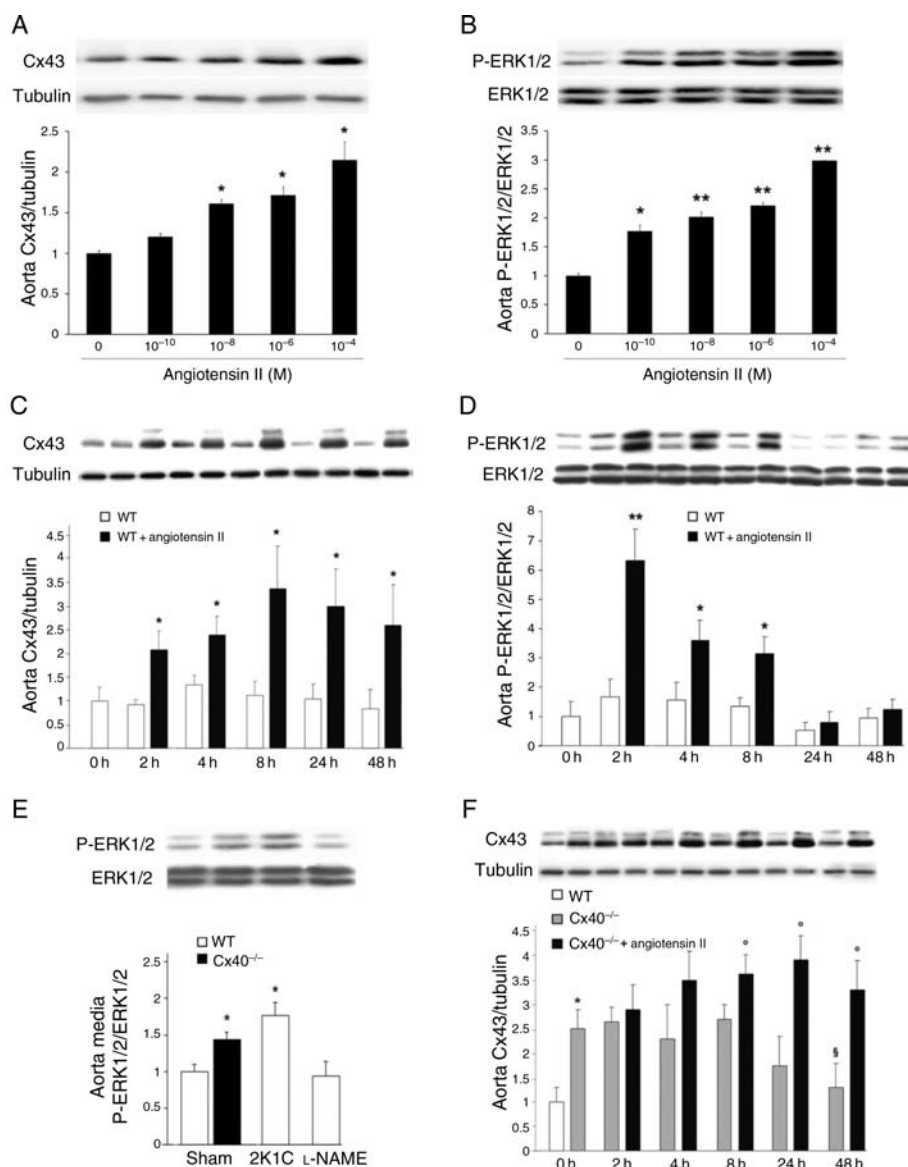


Figure 3 AngII stimulates Cx43 expression in a dose-dependent manner. (A and B) Western blots of WT aortas exposed for 4 h to AngII showed that the peptide increased the levels of Cx43 and P-ERK1/2, at physiologically nanomolar concentrations, and reproduced the two-fold Cx43 increase observed *in vivo*, at concentrations $>0.1 \mu\text{M}$. (C and D) Exposure of WT aortas to $2 \mu\text{M}$ AngII increased within 2 h Cx43 (C), and P-ERK1/2 (D). (E) The levels of the P-ERK1/2 increased in aortas of Cx40^{-/-} and 2K1C mice, but not in those of L-NAME-treated mice. (F) A smaller and delayed effect was observed with aortas of Cx40^{-/-} mice. In the absence of AngII, the levels of Cx43 decreased in this tissue to reach control values within 48 h. Data represent mean + SEM of three experiments. * $P < 0.05$, ** $P < 0.01$, [§] $P < 0.05$ vs. respective untreated WT or Cx40^{-/-} aortas. [§] $P < 0.05$ vs. untreated Cx40^{-/-} aortas at $t = 0$ h.

3.4 The AngII-induced regulation is seen in smaller arteries

Using carotid and mesenteric arteries, which express a Cx pattern analogous to that of the aorta (Figure 4A), we also observed increased levels of Cx43 and ERK1/2 phosphorylation in Cx40^{-/-} mice (Figure 4B). When exposed *in vitro* to AngII, the two types of WT vessels featured an increased expression of Cx43 and phosphorylation of ERK1/2 (Figure 4C), as observed in the aorta (Figure 3C and D). This change was significantly higher in the small-diameter mesenteric arteries than in the larger carotids (Figure 4C). These experiments show that chronic hypertension similarly targets the aorta and the smallest arteries we could test in mice.

3.5 A7r5 cells represent a convenient model of aortic SMCs

To further investigate the molecular mechanism of the AngII induction of Cx43, we searched a cell line that would mimic the main features of primary SMCs. We found that A7r5 expressed a Cx pattern alike that of aortic SMCs (Figure 5A, C, D) and responded to physiologically relevant concentrations of AngII with a similar increase in Cx43 and phosphorylated ERK1/2 (P-ERK1/2) (Figure 5A and B). At a $2 \mu\text{M}$ concentration, which induced in A7r5 cells a two- to three-fold increase in Cx43 similar to that observed *in vivo* and in isolated aortas, A7r5 cells also increased the expression of Cx43, but not that of Cx37, Cx40, and Cx45 (Figure 5C and D) and showed a rapid,

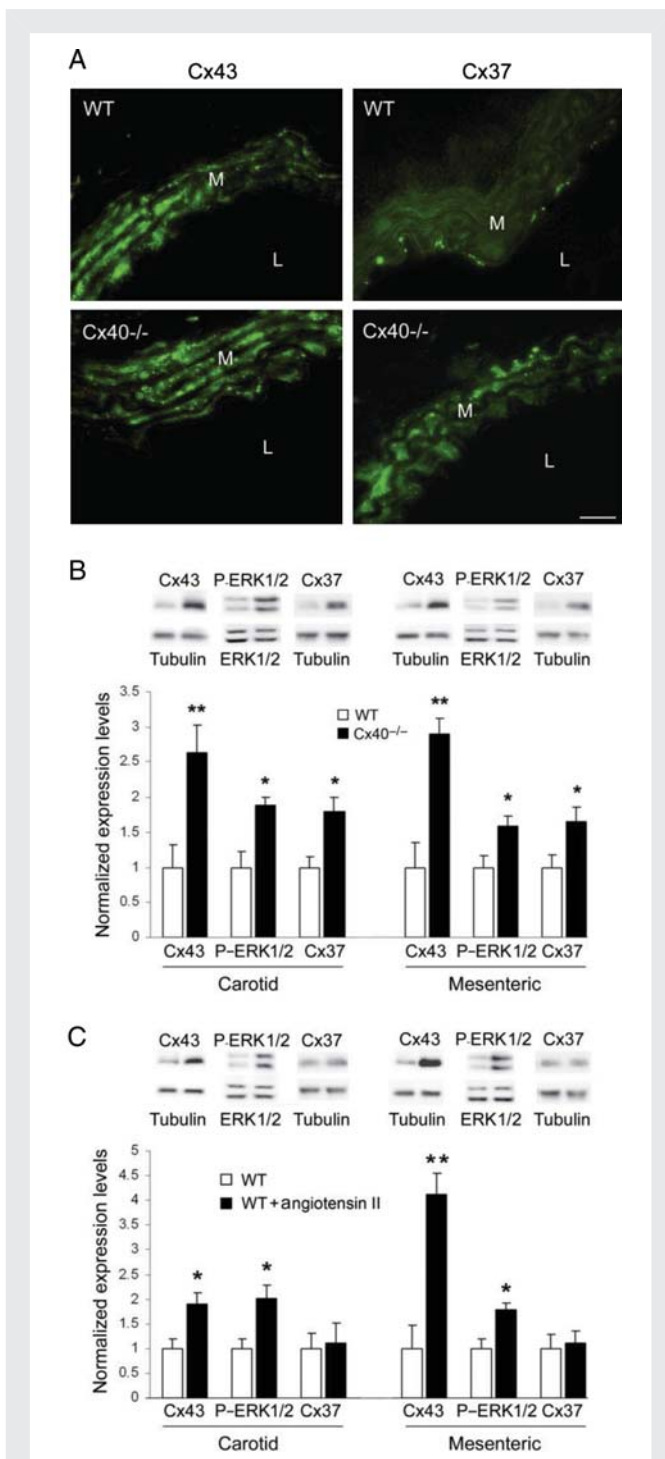


Figure 4 Carotid and mesenteric arteries show a Cx pattern and responsiveness to AngII similar to those of aorta. (A, left panels) Immunostaining showed punctate Cx43 throughout the carotid media. Increased numbers of immunospots were observed in the media of Cx40^{-/-} mice. (A, right panels) Cx37 was detectable in the media of hypertensive but not control mice. L, lumen; M, media. Bar represents 10 μ m. (B) Western blots showed that Cx37, Cx43, and P-ERK1/2 were increased in carotid and mesenteric arteries of Cx40^{-/-} mice. (C) After a 4 h exposure of WT carotids and mesenteric arteries to 2 μ M AngII, Cx43 and P-ERK1/2, but not Cx37, were increased. Data represent mean + SEM of three experiments. * $P < 0.05$, ** $P < 0.01$ vs. respective untreated vessels or WT animals.

AngII-dependent activation of the ERK pathway (Figure 5E). Thus, A7r5 cells reproduced the main Cx and signalling changes observed in native aortas.

3.6 AngII increases Cx43 via activation of the NF- κ B pathway

A7r5 cells exposed to AngII increased the proportion of phosphorylated I κ B α ^{36,37} (Figure 6A and B), with a time course consistent with that of the Cx43 changes (Figure 5A). Immunostaining demonstrated that AngII induced the nuclear translocation of the p65-NF- κ B transcription factor (Figure 6C). Candesartan, and the inhibitor of the ERK1/2 pathway PD98059, prevented the AngII-dependent induction of Cx43 expression, and ERK1/2 and I κ B α phosphorylation (Figure 6B). These results suggest that AngII up-regulates Cx43 via the activation of the ERK and NF- κ B pathways.

3.7 NF- κ B binds to the promoter of the Cx43 gene and activates its transcription

We found a canonical κ B site between -312 and -301 bp of the promoter of the rat *Gja1* gene. Therefore, we transfected A7r5 cells with a luciferase cDNA, under control of two fragments of the rat Cx43 promoter, each containing a κ B site. After 4 h in the presence of 2 μ M AngII, we observed an increase in luciferase activity, whether the reporter gene was driven by the short (258 bp) or long (699 bp) promoter fragment (Figure 6D). This increase was prevented by mutating the κ B site (Figure 6D).

We incubated chromatin from control and AngII-stimulated A7r5 cells with antibodies against either the P65 or P50 form of NF- κ B. Both antibodies immunoprecipitated fragments which, by PCR, yielded a sequence corresponding to -433 and -187 bp (relative to the transcription initiation site³⁸) of the Cx43 promoter (Figure 6E). After a 2 h exposure to AngII, the recovery of this fragment increased in the presence, but not in the absence, of antibodies against NF- κ B (Figure 6E). These data document that AngII enhances the binding of NF- κ B to the *Gja1* promoter.

4. Discussion

The multiple Cxs which are expressed by vascular ECs and SMCs^{4,6} have been implicated in the physiological function of arteries,³⁹ and their adaptation to chronic hypertension.^{22,40} Still, it is not clear whether and how all vascular Cxs are similarly affected by all forms of hypertension.⁴¹ Comparing models of renin-dependent⁵ and -independent hypertension,^{5,7,10,18} we now show that all hypertensive mice featured a thickening of the media, which was associated with increased levels of Cx40 in the intimal ECs, and of Cx37 and Cx45 in the media SMCs. In contrast, Cx43 was up-regulated in the latter layer, without obvious changes in its phosphorylation, only under conditions of renin-dependent hypertension. These observations document that multiple Cx changes are associated with the remodelling of the arterial wall which occurs in response to the altered mechanical and hormonal environment of hypertension. They further show that although the control of Cx37, Cx40, and Cx45 expression does not depend on the circulating levels of renin and AngII, these hormones are required for the control of Cx43. Given that AngII ultimately mediates the vasoconstriction and SMC changes⁴² that result from RAS activation,³¹ we have investigated the effects of this peptide. Consistent with previous data in rats and cultured SMCs,⁴² we have found

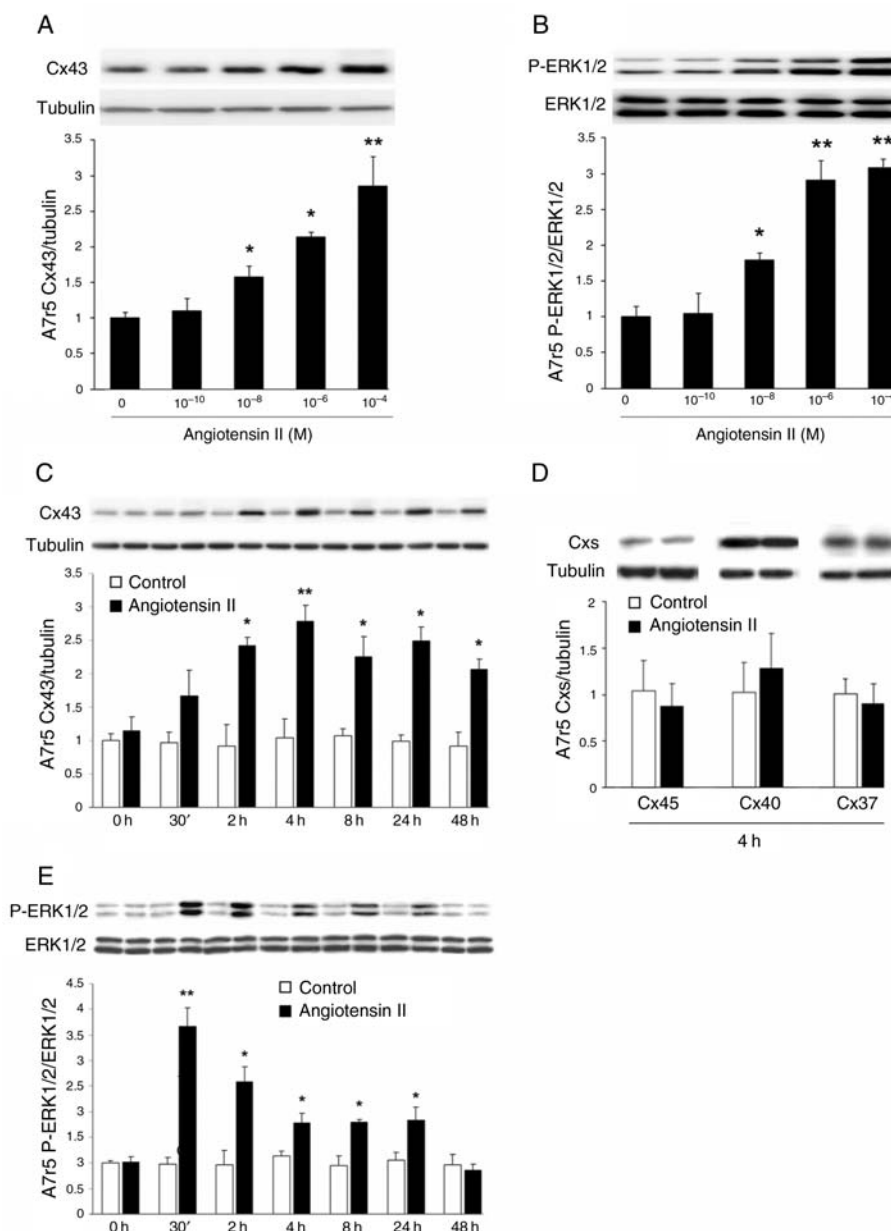


Figure 5 A7r5 cells feature the same Cx43 and signalling changes observed in aortic SMCs. (A and B) Western blots of A7r5 cells exposed for 2 h to increasing concentrations of AngII showed that the peptide increased the levels of Cx43 and P-ERK1/2 at concentrations similar to those that elicited comparable changes in aortic SMCs. (C and D) Western blots showed that A7r5 cells expressed Cx43, Cx37, Cx40, and Cx45. Two-micromolar AngII increased Cx43, but not Cx37, Cx40, and Cx45. (E) AngII also increased the levels of P-ERK1/2. Results are means + SEM of three experiments. * $P < 0.05$, ** $P < 0.01$ vs. untreated cells.

that within a few hours, physiologically relevant levels of AngII increased the expression of Cx43, but not of Cx37, Cx40, and Cx45. This change is induced by the activation of ERK1/2 which, by causing the phosphorylation of I κ B α ,^{43,44} results in the nuclear translocation of NF- κ B. We now document that NF- κ B directly and specifically binds *in situ* to the promoter of the *Gja1* gene which codes for Cx43, and that the NF- κ B binding is enhanced by AngII. The Cx43 changes are rapidly induced by the ERK1/2 activation, but persist even after the ERK activity has returned to basal levels, indicating that other mechanisms contribute to the sustained elevation of Cx43 expression.

These experiments provide the first coherent explanation of how a renin increase enhances Cx43: (i) the activation of RAS increases the circulating levels of AngII; (ii) elevated AngII results in peripheral vasoconstriction, causing hypertension, and triggering SMC hypertrophy and proliferation; (iii) in parallel, AngII promotes, via phosphorylation of ERK1/2, the binding of NF- κ B to the *Gja1* promoter; (iv) this binding trans-activates the *Gja1* gene, leading to increased production of Cx43 in the SMCs (see Supplementary material online, Figure S4). The finding of comparable Cx43 changes in the aorta, the carotids, and the small mesenteric arteries suggests that our observations apply to the many vascular compartments targeted by hypertension.^{15,45}

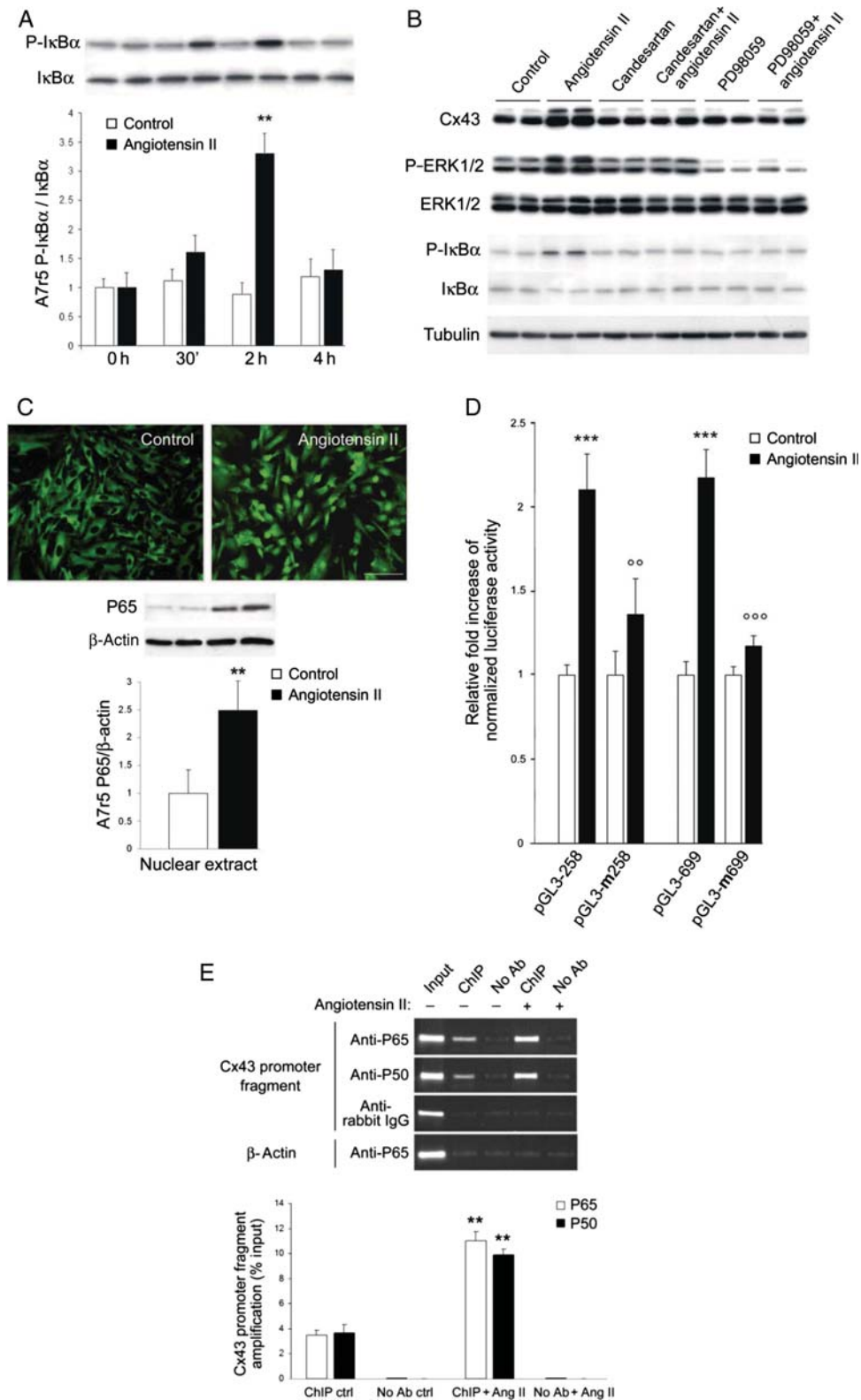


Figure 6 The AngII-induced increase in Cx43 requires the activation of ERK and NF- κ B pathways. (A) Within 2 h, AngII increased the phosphorylation of I κ B α in A7r5 cells. Results are means + SEM of three experiments. $**P < 0.01$ vs. untreated cells. (B) Within 2 h, AngII also increased Cx43, P-ERK1/2, and P-I κ B α levels. Both candesartan and PD98059 prevented these effects. (C) After 4 h, 2 μ M AngII induced a translocation of the P65 phosphorylated form of NF- κ B into the nucleus of A7r5 cells. Bar, 20 μ m. (D) AngII increased luciferase activity of A7r5 cells transfected with either a short or long *Gja1* promoter fragment. Mutation of the NF- κ B motif in either one of the two promoter fragments prevented the increased in the luciferase activity observed after AngII stimulation. (E) Quantitative RT-PCR showed that AngII increased the amount of the *Gja1* promoter fragment, recovered using antibodies against either the P65 or the P50 forms of NF- κ B. Results are means + SEM of three experiments. $**P < 0.01$, $***P < 0.001$ vs. untreated cells; $^{\circ}P < 0.01$, $^{\circ\circ}P < 0.001$ vs. AngII-treated cells.

Our data further implicate that other mechanisms, possibly dependent on the mechanical forces that impinge on the arterial walls during hypertension,^{1,46,47} in the control of Cx37, Cx40, and Cx45, as well as in the sustained elevation of Cx43, once the activation of ERK1/2 ends.

The significance of the increase in Cx43 in renin-dependent hypertension remains to be elucidated. Beside its major vasoconstrictor effect, AngII also stimulates vascular remodelling by promoting SMC growth and the production of extra cellular matrix, which contribute to increased aortic stiffness,⁴⁸ a frequent consequence of hypertension.⁴⁹ Increased Cx43 expression in the presence of elevated AngII may therefore contribute to regulate the elasticity of the vascular wall. If so, the pharmacological targeting of Cx43 may be therapeutically useful, in addition to the standard anti-hypertensive drugs which aim at counteracting the AngII effects.⁵⁰

In summary, by comparing control and Cx40-lacking mice, under conditions of renin-dependent (2K1C) and renin-independent hypertension (L-NAME administration), we have found that Cx37, Cx40, and Cx45 are up-regulated in the wall of different types of arteries, probably as a result of the increased mechanical forces impinging on the vessels' wall. In contrast, Cx43 was up-regulated only in the renin-dependent model of hypertension, due to an AngII-dependent mechanism, which specifically activates the transcription of the Cx43 gene. These findings document that the expression of several vascular connexins is affected by chronic hypertension, though by different mechanisms, and in different compartments of the arterial wall.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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