# Chronic hypertension alters the expression of Cx43 in cardiovascular muscle cells

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## Abstract

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Connexin43 (Cx43), the predominant gap junction protein of muscle cells in vessels and heart, is involved in the control of cell-to-cell communication and is thought to modulate the contractility of the vascular wall and the electrical coupling of cardiac myocytes. We have investigated the effects of arterial hypertension on the expression of Cx43 in aorta and heart in three different models of experimental hypertension. Rats were made hypertensive either by clipping one renal artery (two kidney, one-clip renal (2K,1C) model) by administration of deoxycorticosterone and salt (DOCA-salt model) or by inhibiting nitric oxide synthase with $N^G$-nitro-L-arginine methyl ester (L-NAME model). After 4 weeks, rats of the three models showed a similar increase in intra-arterial mean blood pressure and in the thickness of the walls of both aorta and heart. Analysis of heart mRNA demonstrated no change in Cx43 expression in the three models compared to their respective controls. The same 2K,1C and DOCA-salt hypertensive animals expressed twice more Cx43 in aorta, and the 2K,1C rats showed an increase in arterial distensibility. In contrast, the aortae of L-NAME hypertensive rats were characterized by a 50% decrease in Cx43 and the carotid arteries did not show increased distensibility. Western blot analysis indicated that Cx43 was more phosphorylated in the aortae of 2K,1C rats than in those of L-NAME or control rats, indicating a differential regulation of aortic Cx43 in different models of hypertension. The data suggest that localized mechanical forces induced by hypertension affect Cx43 expression and that the cell-to-cell communication mediated by Cx43 channels may contribute to regulating the elasticity of the vascular wall.

## Introduction

Gap junctions are seen at sites where the plasma membranes of two adjacent cells become closely apposed. In these regions, the two interacting membranes feature specialized microdomains characterized by the concentration of large protein assemblies named connexons. These structures provide the wall of specialized intercellular channels that allow for the passage of ions, as well as for the exchange from one cell to another of metabolites and second messengers with a mass up to 1 kDa (1,2). Gap junction channels are formed by the hexameric assembly of membrane-spanning proteins, known as connexins, which in mammals belong to a family of 14 members (3). Five of these proteins, referred to as Cx43, Cx45, Cx46, Cx40 and Cx37, have been identified in the cardiovas-

cular system (4). As yet, little is known about their physiological role and their possible changes in cardiovascular diseases.

Gap junctions ensure the electrical and mechanical coupling of different types of muscle cells (5,6). Such a role is critical in the heart, since proper propulsion of blood in the circulation obrigatorily depends on the coordinated contraction of both atrial and ventricular cardiomyocytes (7,8). This contraction in turn is mediated by the rapid propagation of action potentials to multiple cells that should depolarize in coordination. These events are dependent on gap junctional communications, whereby adjacent cardiomyocytes exchange current-carrying ions. By diffusing from one cell to the next, these ions synchronize the electrical and mechanical activity of neighboring cells (7). Coordination of smooth muscle cells of the vascular wall is also critical to the local modulation of vasomotor tone, thus contributing to the proper function of large vessels. The aorta, which is a sparsely innervated and electrically quiescent vascular tissue, is likely to be particularly dependent on gap junctional communications for coordinating the responses of smooth muscle cells to diverse neural and endothelial signals (9,10).

Characterization of the expression of the proteins that form gap junctions may provide insights into the possible involvement of junctional channels during cardiac and arterial hypertrophy associated with hypertension (11-13). Thus, conditions perturbing the function of the aortic wall, as observed during chronic hypertension, are expected to be associated with alterations of connexins, gap junctions or coupling.

To test this hypothesis, we have studied the expression of Cx43, the physiologically predominant connexin of myocardial and aortic smooth muscle cells (14), during chronic hypertension. To this end, we have investigated three rat models characterized by a similar degree of hypertension and by hypertrophy of both aortic and heart walls,

but differing markedly by the mechanism causing these changes. In the mineralocorticoid salt-induced model (deoxycorticosterone, DOCA-salt), hypertension resulted from increased retention of sodium in the presence of suppressed renin secretion (15,16). In the two kidney, one-clip model (2K,1C), hypertension was produced by clipping one renal artery, leading to stimulation of renin secretion and to an angiotensin II (AngII)-dependent elevation of blood pressure. Under the conditions provided by these two models, the isobaric distensibility of the carotid has been shown to increase due to a reduced elastic modulus, corresponding to a reduction of wall material stiffness (17,18). These biomechanical changes are not observed in a third rat model, in which a degree of hypertension similar to that observed in the two kidney, one-clip and the DOCA-salt models can be induced by inhibiting nitric oxide synthase (NOS) with $N^G$-nitro-L-arginine-methyl ester (L-NAME) (19,20). In this model, hypertension is associated with limited cardiovascular hypertrophy (20,21), and with no increase in the isobaric distensibility of the carotid (22). Comparison of the three models is therefore of interest to determine whether Cx43 changes are consistently associated with hypertension and whether they vary, qualitatively and/or quantitatively, as a function of the mechanisms that lead to increased blood pressure and to cellular changes in the aortic wall (23).

## Results

### Effects of treatments on blood pressure

Blood pressure levels were 1.4-1.6-fold higher in the 2K,1C, DOCA-salt and L-NAME animals compared to control (Figure 1).

### Effects of hypertension on the heart

Hypertensive rats of the 2K,1C, and

DOCA-salt models showed a 30% increase in heart index (i.e., weight of myocardium per unit animal weight) compared to their normotensive counterparts (Figure 2). In agreement with this change, Northern blot analysis showed a two-fold increase in the expression of α-skeletal actin mRNA in the two groups of hypertensive rats and histology revealed a thickening of the left ventricular wall (11). Hearts of L-NAME hypertensive rats were also hypertrophied compared to those of normotensive controls, as indicated by a 17% increase in cardiac weight index (Figure 1). In agreement with this change, Northern blot analysis of heart tissue showed a three-fold increase in the expression of atrial natriuretic peptide (ANP) mRNA and a two-fold increase in the expression of skeletal α-actin mRNA in the hypertensive rats (23).

Quantitative assessment by Northern blot analysis showed that Cx43 expression was similar in the hypertrophied hearts of hypertensive rats of the three models studied, and in those of normotensive controls (Figure 2). This finding was correlated with the absence of significant differences in the levels and distribution of Cx43 which was immunola-

beled in the intercalated discs of heart muscle in the three models studied (11,23).

## Effects of hypertension on the aorta

The thickness of the intima plus media layers of aorta was significantly larger in the 2K,1C and DOCA-salt hypertensive rats than in normotensive animals, resulting, in spite of a constant lumen radius, in a 40% increase of the vessel cross-sectional area (CSA), which was given by the formula: CSA = $\pi$ [(lumen radius + media-intima thickness)$^2$ - (lumen radius)$^2$] (Figure 3). These changes were due to an enlargement of smooth muscle
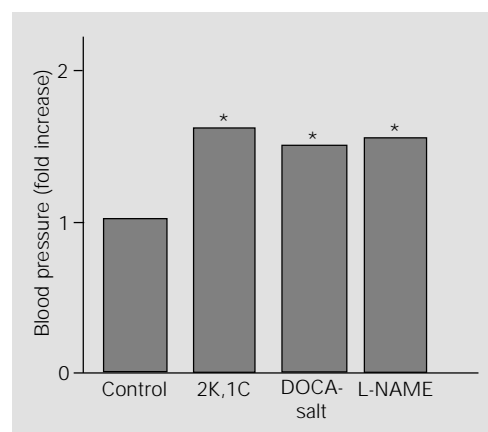


Figure 1 - Increase in blood pressure in the 2K,1C-, DOCA-salt- and L-NAME-treated rats. In the three models studied, blood pressure was found to be increased 1.4-1.6-fold over control values (*P<0.05) four weeks after the beginning of treatment (Bonferroni-Dunn test).
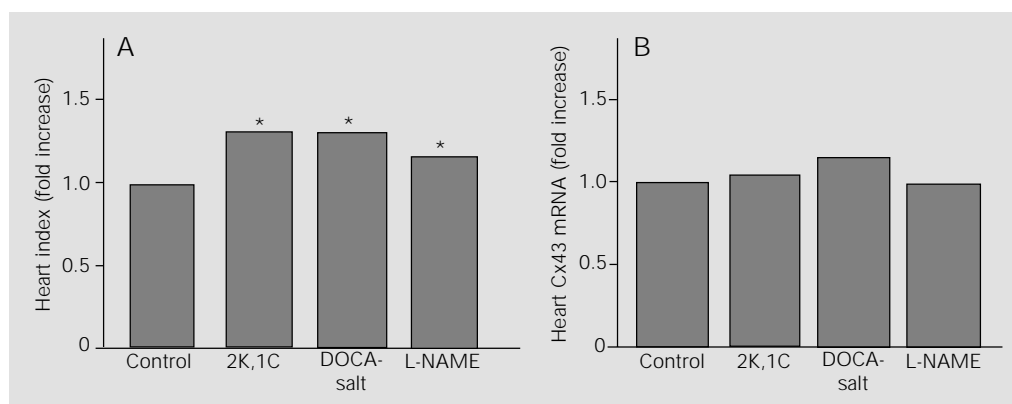


Figure 2 - Thickening of the cardiac wall and expression of Cx43 in hearts of hypertensive rats. A, As compared to normotensive animals, the 2K,1C and DOCA-salt hypertensive rats showed a similar 30% increase in heart index. In L-NAME rats, this increase was only 17%, on average. Values are reported as means of about 10 measurements (one measurement per rat) compared to the control value which was set at 1. *P<0.01 compared to control (Fisher's protected least significant difference test). B, Analysis of heart RNA revealed that the levels of the Cx43 transcript, which was mostly provided by cardiomyocytes, were not altered in the three types of hypertensive rats investigated. Values represent ratios of densitometric measurements of Cx43 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs, and are reported as means of about 10 measurements, relative to the control value, which was given a value of 1.

cells, whose numerical density was slightly reduced, and were paralleled by a significant increase in the expression of α-skeletal actin. In comparison to controls, the L-NAME hypertensive animals also showed a 25% thickening of the aortic wall (intima plus media) resulting in an increased cross-sectional area of the aorta (Figure 3).

Quantitative assessment of Cx43 gene expression by Northern blotting of total aorta RNA, which was mostly contributed to by smooth muscle cells, showed significantly higher values in the aorta of 2K,1C and DOCA-salt hypertensive rats than in that of normotensive rats (Figure 3). In contrast, when compared to controls, the transcript of Cx43 was reduced by about 50% in the aorta of L-NAME-treated animals (Figure 3).

Cx43 was immunolocated on smooth muscle cells of the aortic media in both hypertensive and normotensive rats. The number of immunofluorescence spots reflecting the abundance of Cx43 was larger in the aorta of control rats than in that of L-NAME-treated rats. In contrast, the smooth muscle cells of 2K,1C and DOCA-salt rats showed a modest but sizeable increase in the amount of Cx43, as monitored by immunolabeling of aorta cryosections (11).

Western blot analysis of total proteins extracted from the aorta showed that Cx43 was significantly decreased in L-NAME hypertensive rats. Extracts from the aorta of these animals contained one major immunoreactive band (of about 44 kDa) whose average intensity was less than 50% that of controls. In contrast, extracts from aortas of 2K,1C hypertensive rats contained three immunoreactive bands indicating that Cx43 was consistently more phosphorylated in the aorta of these animals than in that of both controls and L-NAME animals. Also the levels of Cx43 in 2K,1C were significantly higher than in controls (23).

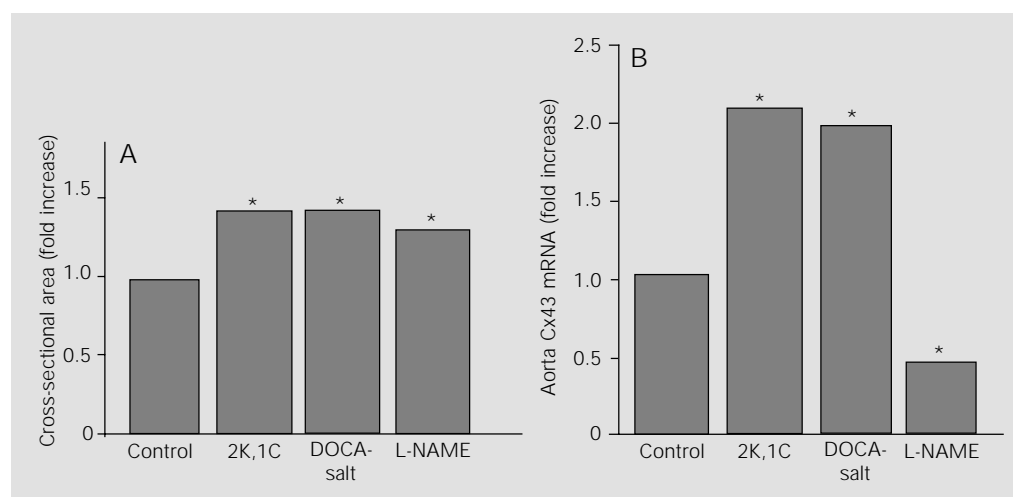A trend towards a reduced distensibility-pressure curve for the carotid artery was



Figure 3 - Thickening of the aortic wall in hypertensive rats and expression of Cx43 in aortae of hypertensive rats. A, After four weeks of hypertension, the aorta of two kidney, one-clip renal (2K,1C) and DOCA-salt hypertensive animals showed a thickened wall, resulting in a 40% increase in the cross-sectional area (control value was set at 1), despite a constant internal diameter. The aorta of L-NAME hypertensive rats was thickened to a lesser extent, resulting in a 25% increase in cross-sectional area. Values are reported as means of 4-8 measurements. *P<0.01 compared to control (Scheffé test). B, Analysis of aorta RNA revealed that the transcript for Cx43, which was mostly provided by smooth muscle cells, was increased about two-fold in the hypertensive rats of the 2K,1C and DOCA-salt models. In contrast, the hypertensive rats treated with L-NAME showed a 50% reduction of the Cx43 transcript compared to the control value (which was set at 1 in all groups). Values represent ratios of densitometric measurements of Cx43 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs, and are reported as means of 6-10 experiments. *P<0.01 compared to control (Scheffé test).

seen in the L-NAME-treated rats, in the range of blood pressures (120-140 mmHg) that could be compared. In contrast, carotid distensibility has been shown to be markedly increased in the 2K,1C model (20,22).

## Discussion

We have examined the effects of chronic hypertension on the expression of Cx43, the major native connexin of the cardiovascular system, in three different experimental rat models.

After one month, the increase in blood pressure achieved in the three models was comparable. We found that all hypertensive animals exhibited cardiac hypertrophy (11) in the absence of differences in the levels of Cx43 connecting myocardial cells. This finding suggests that Cx43 is not involved in the myocardial adaptation that accompanies a hypertension-induced increase in heart load. This conclusion does not rule out that other connexins such as the Cx45, Cx40, and Cx37 isoforms (24) may participate in the heart changes induced by hypertension. Indeed, the inactivation of the Cx43 gene in transgenic mice suggests that, at least under certain conditions, Cx43 may be functionally replaced by other connexins (25).

Under the conditions used in the present study, all hypertensive animals also exhibited a thickening of the aortic wall, which was mostly accounted for by the hypertrophy of smooth muscle cells and the accumulation of extracellular materials. The 2K,1C and DOCA-salt rats also exhibited a comparable increase in the level of Cx43 that was expressed by the smooth muscle cells of the aortic media. In contrast, decreased levels of Cx43 were found in the same cells of L-NAME-treated rats.

In the 2K,1C model, the development of hypertension results from the constriction of one renal artery and the ensuing activation of the renin-angiotensin system, as reflected by enhanced renin mRNA levels in the hypo-perfused kidney and by elevated plasma renin activity (26). The further proteolytic cleavage of angiotensinogen by renin and the processing of angiotensin I by angiotensin-converting enzyme leads to the generation of the biologically active AngII. AngII is a most potent vasoconstrictor peptide which also plays a role in the development of vascular and cardiac hypertrophy (27,28). Therefore, the cellular and connexin changes observed in the 2K,1C rats could be due to both the increased blood pressure and the increased levels of AngII. To discriminate between these possibilities, we have studied the DOCA-salt model, which is characterized by the functional suppression of the renin-angiotensin system. In this model, hypertension is induced by administration of a salt-retaining mineralocorticoid in association with a high sodium intake (15). The finding in this model of vascular and connexin changes similar to those observed in the 2K,1C model indicates that these changes could not be related to the circulating levels of AngII, which differed considerably in the 2K,1C and the DOCA-salt hypertensive rats and, hence, are likely to be associated with the elevation of blood pressure. The molecular mechanism leading to the pressure-induced increase in the expression of the Cx43 gene remains to be elucidated. The presence of multiple promoters in the 5' untranslated region of this gene (29,30) raises the possibility that the tissue-specific regulation of this increase observed here is controlled by distinct transcription factors (31). Of particular interest in this context is the recent finding that transcription of the Cx43 gene may be promoted by an increase in the expression of c-fos (32), since the mRNA coding for this transcription factor accumulates in smooth muscle cells of rat aortas following exposure to angiotensin II (33,34), which contributes to hypertension in the 2K,1C model.

When exposed to chronic hypertension, conduit arteries undergo profound functional

and structural changes characterized by an outward hypertrophic remodeling with preserved isobaric luminal diameter (18,35). This remodeling, which results from hypertrophy of smooth muscle cells and alterations of extracellular matrix, may be regarded as an adaptation to normalize wall stress. However, this adaptation is also likely to modify the mechanical properties of arteries, which could be detrimental in the long term (18). Previous studies have shown that the distensibility and compliance of various arteries are increased under isobaric conditions in the 2K,1C hypertensive animals (18,20), suggesting that changes in tissue composition and architecture permit arteries to maintain adequate elastic properties in spite of increased blood pressure. In contrast, the hypertension caused by inhibition of nitric oxide is not associated with an increase in the isobaric distensibility of the carotid artery, despite a thickening of the arterial wall which is similar to that observed in other experimental models of hypertension. The different viscoelastic properties of arteries in the 2K,1C and L-NAME-treated rats implies a differential structural and/or functional organization of tissues making up the wall of resistance arteries. Thus, the L-NAME-induced hypertension was associated with a decrease in the expression of Cx43 in the smooth muscle cells of the aorta, contrasting with the findings for both the 2K,1C and the DOCA-salt models (11,12). The significance of the decrease in Cx43 in the L-NAME-treated rats remains to be elucidated. Several electrophysiological studies have suggested that gap junction proteins may be important to coordinate the mechanical contractions of smooth muscle cells, possibly to insure a proper modulation of the vasomotor tone of the aortic wall (9). Certainly, Cx43 can provide an intercellular pathway for the syncytial functioning of distant smooth muscle cells that could be recruited for synchronous contraction through propagation of gap junction-permeant second messengers (36).

Eventually, a different post-translational regulation of Cx43 was observed in the aortic smooth muscle cells of the different hypertension models. Thus, whereas the degree of Cx43 phosphorylation was found to be increased in the 2K,1C animals, it was decreased in the L-NAME-treated rats, which essentially expressed a non-phosphorylated form of Cx43. Since connexin phosphorylation can affect the extent of junctional communication, this difference could result in a selective cell-to-cell exchange of the molecules involved in both hypertrophy (2K,1C model) and polyploidy (L-NAME model) of vascular smooth muscle cells. Blockade of the nitric oxide production by endothelial cells after treatment with L-NAME is expected to decrease the apoptosis and to promote the proliferation of smooth muscle cells (37,38), thus accounting for their accumulation and polyploidy on the aortic wall. The reduced expression of Cx43 in the aorta of L-NAME-treated rats may also contribute, as in atherosclerotic lesions (39), to upregulating the adhesion of monocytes/macrophages to the aorta. After inhibition of NO production by L-NAME treatment, this adhesion increases (40).

In summary, we have found that the expression of Cx43 is differentially regulated in the hypertrophic muscle cells of heart and aorta, and that this differential regulation takes place in rats made similarly hypertensive by different mechanisms. Although further studies are needed to understand how the changes in Cx43 expression participate to the adaptive response of the aorta to high blood pressure, our data indicate that Cx43 may represent a suitable, tissue-specific marker to monitor hypertension-induced changes in the vasculature. The altered expression and phosphorylation of Cx43 in the aorta of hypertensive rats raises the possibility that this gap junction protein may contribute to the lack of autoregulation of arterial distensibility.

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