Endothelium-dependent desensitization to angiotensin II in rabbit aorta: the mechanisms involved

S. Jerez, M. Peral de Bruno, and A. Coviello

Abstract: The aim of this study was to characterize the role of the endothelium in angiotensin II-desensitization and its mechanisms of action. Rabbit aortic rings were exposed to increasing doses of angiotensin II (Ang II, 10^{-9} to 2.5×10^{-6}) to generate two cumulative dose-response curves (CDRC I and II). A 50-min interval separated CDRC I and II. Desensitization was observed at all doses in unrubbed aortic tissue and at lower doses in rubbed aortic tissue. Tachyphylaxis was greater in arteries with endothelium. Treatment of intact rings with L-N^G-nitroarginine methyl ester (L-NAME, 10^{-4} M) did not prevent this phenomenon. However, indomethacin (10^{-5} M) and miconazol (10^{-6} M) attenuated Ang II-desensitization. Treatment of unrubbed rings with nifedipine (10^{-6} M) and cromakalim (10^{-6} M) inhibited the effect of indomethacin. To confirm the involvement of K^+ channels, unrubbed and rubbed aortic rings were treated with the K_{Ca}^{2+} blockers apamin (10^{-7} M), tetraethylammonium (TEA, 10^{-3} M), and iberiotoxin (10^{-8} M), and the K_{ATP} blocker glibenclamide (10^{-5} M). In both arteries apamin, TEA, and glibenclamide abolished the tachyphylaxis without changes in the maximal response. Iberiotoxin diminished Ang II-desensitization in rubbed but not unrubbed arteries. Results from this study suggest that Ang II-desensitization involves endothelium-dependent and -independent mechanisms. Endothelium-dependent desensitization could be mediated by a cyclooxygenase-cytochrome P_{450} product, which could act by increasing K_{Ca}^{2+} channel activity.

Key words: angiotensin II, rabbit aorta, desensitization, endothelium, cyclooxygenase products.

Résumé : La présente étude a eu pour but de caractériser le rôle de l'endothélium dans la désensibilisation induite par l'angiotensine II ainsi que les mécanismes qui seraient mis en cause. On a enregistré la tension isométrique produite par des anneaux aortiques de lapins. On a obtenu deux courbes dose-réponse cumulatives à intervalles de 50 min. On a observé une désensibilisation pour toutes les doses dans les aortes pourvues d'un endothélium et pour les doses plus faibles dans les aortes dépourvues d'endothélium. La tachyphylaxie a été plus forte dans les artères pourvues d'un endothélium. Le traitement des anneaux intacts au moyen de L-NAME (10^{-4}) n'a pas prévenu ce phénomène. Toutefois, l'indométhacine (10^{-5} M) et le miconazol (10^{-6} M) ont atténué la désensibilisation induite par l'angiotensine II. Le traitement des anneaux pourvus d'un endothélium au moyen de nifédipine (10^{-6} M) et de cromakalim (10^{-6} M) a inhibé l'effet de l'indométhacine. Pour confirmer la participation des canaux K^+, on a traité les anneaux aortiques pourvus et dépourvus d'endothélium avec les bloqueurs de K_{Ca}^{2+}, apamine (10^{-7} M), tetraethylammonium (TEA, 10^{-3} M), ibériotoxine (10^{-8} M), et le bloqueur de K_{ATP}, glibenclamide (10^{-5} M). Dans les deux artères, l'apamine, le TEA et le glibenclamide ont supprimé la tachyphylaxie, sans modifier la réponse maximale. L'ibériotoxine a diminué la désensibilisation induite par l'angiotensine II dans les artères dépourvues d'endothélium mais pas dans les artères pourvues d'un endothélium. Ces résultats donnent à penser que la désensibilisation induite par l'angiotensine II a mis en cause des mécanismes dépendants et indépendants de l'endothélium et que les premiers seraient véhiculés par un produit cyclooxygénase-cytochrome P_{450} qui pourrait agir en augmentant l'activité des canaux K_{Ca}^{2+}.

Mots clés : angiotensine II, aorte de lapin, désensibilisation, endothélium, produits de la cyclooxygénase.

[Traduit par la Rédaction]

Introduction

Desensitization (tachyphylaxis) is defined as a decrease in cellular responsiveness to a hormone following prolonged or repetitive exposure (Hausdorff et al. 1990; Bouvier et al. 1995). Desensitization been observed for angiotensin II (Ang II) in some tissues such as the rat vascular mesenteric bed (Fasciolo and Binia 1981), rat aorta (Khairallah et al. 1966; Kuttan and Sim 1993), and rabbit celiac and mesenteric arteries (Aiken 1974). In isolated rabbit aorta,

Received July 24, 2000. Published on the NRC Web site on XXXX, 2000.

S. Jerez[1] and A. Coviello. Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán, Argentina, and Fundación INELCO.
M. Peral de Bruno. INSIBIO, Universidad Nacional de Tucumán, Argentina, and Fundación INELCO.

[1]Corresponding author at Fundación INELCO, España 3706, 4000, Tucumán, Argentina (e-mail: sjerez@tucbbs.com.ar).

tachyphylaxis is observed in rings (Silva et al. 1988) but not in strips (Aiken 1974). Through Ang II, the renin-angiotensin system plays an important role in maintaining arterial pressure, thus regulating vascular tone. Ang II is a potent vasoconstrictor of vascular smooth muscle (VSM), inducing intracellular calcium increase through specific AT_1 receptors. Investigators have reported a modulating effect of the endothelium in the contractile response of VSM to Ang II (Gruetter et al. 1988). Ang II stimulates endothelial synthesis of vasodilators such as nitric oxide (Zhang et al. 1994; Zhang et al. 1995; Boulanger et al. 1995) and prostaglandins (Lin and Nasjletti 1991), and vasoconstrictors such as endothelin (Webb et al. 1992; Chen et al. 1995) and lipoxygenase-derived eicosanoids (Lin et al. 1994; Takizawa et al. 1998). The influence of the endothelium on the desensitization phenomenon has been described by Gruetter et al. (1987) and more recently by Li et al. (1995). Both studies reported that the presence endothelium increases tachyphylaxis to Ang II in rat aorta; however, this phenomenon persists when the endothelium is destroyed. This means that endothelium-dependent and -independent mechanisms are involved. Different hypotheses have been proposed to explain Ang II-desensitization. Kuttan and Sim (1993) suggested that there are changes at the level of the receptor either in coupling affinity or efficiency, which in turn are regulated by factors released from the endothelium or smooth muscle. More recently, Kai et al. (1996) found down-regulation of protein $G_{\alpha q}/G_{\alpha 11}$ levels after prolonged exposure to Ang II in cultured rat vascular smooth muscle cells (VSMC). However, down-regulation of neither $AT_{1A}R$ nor $G_{\alpha q}/G_{\alpha 11}$ was observed after a short-term pretreatment of Ang II, suggesting that the homologous short-term desensitization may be attributed to receptor uncoupling. Our study was conducted to evaluate the role of the endothelium in Ang II-desensitization and its mechanisms of action

Materials and methods

Rabbit aortic ring preparation

Our work was carried out in accordance with the principles and guidelines of the Canadian Council on Animal Care. Experiments were performed on isolated rabbit thoracic aorta from male Flanders hybrid rabbits (1.5–2.5 kg) obtained from a slaughterhouse. The thoracic aorta was carefully dissected and cleaned of adherent fat and connective tissue. Five-millimetre wide rings were cut and mounted in a 10-mL organ bath containing Krebs solution of the following composition (mM): NaCl 128, KCl 4.7, $NaHCO_3$ 14.4, NaH_2PO_4 1.2, Na_2-EDTA 0.1, $CaCl_2$ 2.5, glucose 11.1, pH 7.2. Krebs solution was kept at 37°C and aerated with 95% O_2 and 5% CO_2.

Isometric contractions were measured using force-displacement transducers and were recorded under an initial tension of 2 g, which had been found to be the optimal tension for KCl-induced contraction (100 mM). All preparations were allowed to equilibrate for 90 min and were washed with Krebs solution at 15-min intervals. The endothelium was kept intact in some rings, but in other groups the endothelium was removed by rubbing the luminal surface. Acetylcholine was used to ensure that the endothelium had been removed. The rings were stimulated with noradrenaline (NA, 5×10^{-6} M) and when the maximal contraction was achieved acetylcholine (10^{-6} M) was added to establish its relaxing effect.

Experimental protocols

Aortic rings were exposed to increasing doses of Ang II (10^{-9} to 2.5×10^{-6} M) at 50-min intervals to construct two dose-response curves (CDRC I and II).

To evaluate the specificity of the endothelium-dependent tachyphylaxis observed with Ang II, aortic rings were exposed to NA. Aortic rings with and without endothelium were treated with 5×10^{-7} M NA or no NA (control) for 15 min followed by one CDRC to Ang II. Rings were rinsed and a 50-min recovery period was allowed prior the next exposure.

To study the role of NO in Ang II-tachyphylaxis, L-N^G-nitroarginine methyl ester (L-NAME, 10^{-4} M), a NO synthase inhibitor, was used in arteries with intact endothelium, and the same dose of D-N^G-nitroarginine methyl ester (D-NAME, the inactive isomer) was used as a control. Both compounds were added to the bath 30 min before CDRC I and maintained during the whole experiment. A similar protocol was used with indomethacin (10^{-5} M), an inhibitor of cyclooxygenase, in arteries with and without endothelium.

To establish whether voltage-dependent Ca^{2+} channels that are senstive to dihidropyridines were involved in this phenomenon, a blocking agent (nifedipine, 10^{-6} M) was added to the bath containing the aorta with endothelium in the presence and absence of indomethacin (experimental and control, respectively) for the 50-min period that followed the first stimulation. After this time, nifedipine was removed by washing before CDRC II. Arteries with endothelium that were not treated with nifedipine were washed for 50 min following CDRC I with Ca^{2+}-free EGTA (3 mM). The Ca^{2+}-Krebs solution was restored before the CDRC II. This protocol was then discarded because the Ang II-contractile response during CDRC II was blunted.

To evaluate the role of K^+ channels, arteries with endothelium were incubated for the 50-min period following CDRC I with a K_{ATP} opener (cromakalim 10^{-6} M). Some of these arteries were treated with indomethacin (10^{-5} M) while others were left untreated. Cromakalim was removed by washing before CDRC II. Similar protocols were performed in rubbed and unrubbed aorta with the Ca^{2+} dependent K^+-channel blockers apamin (10^{-7} M), which blocks the small-conductance voltage-independent channels (Blatz and Magleby 1986; Silva et al. 1994), and TEA (10^{-3} M) and iberiotoxin (10^{-8} M), which inhibit the large-conductance voltage dependent channels (Sadoshima et al. 1988; Nelson and Quayle 1995; Silva et al. 1999). Another experiment was performed using the ATP-sensitive channel blocker glibenclamide (10^{-5} M) (Quayle et al. 1995).

Results are expressed as mg of isometric contraction or a percentage of the maximal contractile force obtained during CDRC Drugs

Human angiotensin II, D-NAME, indomethacin, nifedipine, cromakalim, apamin, tetraethylammonium, and iberiotoxin were purchased from Sigma Chemical Co, St. Louis, Mo. L-NAME, glibenclamide, and miconazol were a gift from Dr Howard Lippton (Tulane Medical School, New Orleans, La.) and Alberto Nasjletti (New York Medical College, Valhalla, N.Y.).

Statistical analysis

Data are presented as mean values ± SEM and were analysed by ANOVA with replications and Duncan's test to evaluate CDRC. The pD_2 (negative log of molar concentration of Ang II inducing 50% of the maximal contraction) and the maximal contractile response were calculated using a curve-fitting analysis program. The Student's t test (paired or unpaired) was used to compare pD_2 values or maximal response. $P < 0.05$ was considered statistically significant (two-tail test).

Fig. 1. Cumulative dose-response curve (CDRC) to Ang II in unrubbed (n = 11) rabbit aortic rings. * $P < 0.05$, ** $P < 0.01$ indicates a statistically significant difference between CDRC I and II (ANOVA and Duncan's test). Each point represents mean ± SEM.

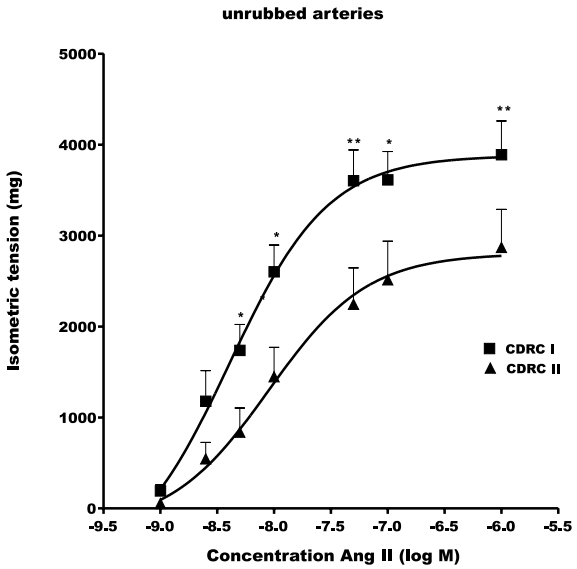
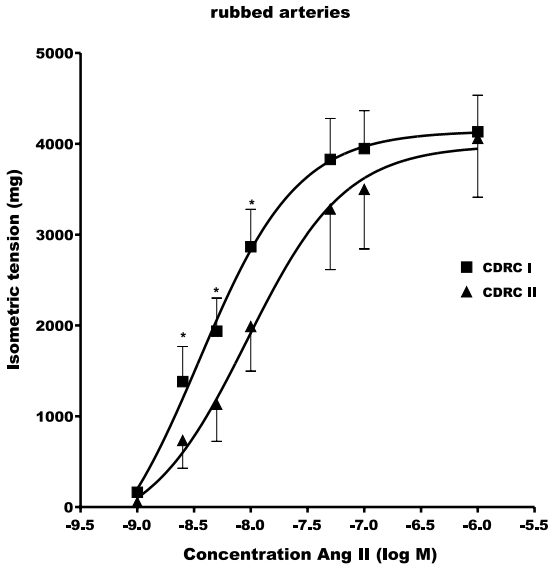


Fig. 2. Cumulative dose-response curve (CDRC) to Ang II in rubbed (n = 9) rabbit aortic rings. * $P < 0.05$ indicates a statistically significant difference between CDRC I and II (ANOVA and Duncan's test). Each point represents mean ± SEM.



Results

Response to Ang II in isolated rabbit aorta

The contractile response to Ang II (10^{-9} to 2.5×10^{-6} M) was dose-dependent. No differences were found in CDRC I between arteries with or without endothelium [ANOVA, not significant (ns)]. Desensitization to Ang II for all doses was observed in aorta with endothelium and the maximal contractile response was blunted (Fig. 1). However, in paired rings without endothelium, desensitization was observed only with lower doses with recuperation of the contractile response at concentrations up to 10^{-8} M after 50 min of washing with Krebs solution (Fig. 2). Tachyphylaxis was greater in arteries with endothelium. There were differences in pD_2 between CDRC I and CDRC II in unrubbed arteries. Likewise, a significant shift to the right of CDRC II was observed in arteries without endothelium. However, no differences in pD_2 were found between arteries with and without endothelium in CDRC I or II (Table 1).

Endothelium-dependent desensitization was not observed with NA. Indeed, the contractile response to NA (5×10^{-7} M) was increased in the second stimulation in rubbed and unrubbed arteries. The treatment with Ang II before the second stimulation did not modify this effect (Table 2).

Treatment with L-NAME

An increase in the maximal contractile response of CDRC I was observed in unrubbed arteries treated with 10^{-4} M L-NAME [5136 ± 735 mg (n = 9) for arteries treated with L-NAME vs 3355 ± 424 mg (n = 9) for controls treated with 10^{-4} M D-NAME, $P < 0.05$, ANOVA). However, no differences were found between pD_2 values (Table 1). This treatment did not modify desensitization to Ang II observed in CDRC II in control aorta (Fig. 3). There were similar differences in pD_2 between CDRC I and II in arteries treated with L-NAME and D-NAME (Table 1).

Effect of indomethacin

Treatment of unrubbed arteries with 10^{-5} M indomethacin did not modify the contractile response of CDRC I with respect to controls (ANOVA, ns) and reversed desensitization for Ang II at doses higher than 10^{-8} M (Fig. 4). However, at the same concentration it had no effect in CDRC II in arteries without endothelium (data not shown). A significant shift to the right of CDRC II was observed, but no differences in pD_2 were found between arteries treated with indomethacin and controls in CDRC I or II (Table 1).

The contractile response observed in CDRC II after treatment with indomethacin in aorta with endothelium was similar to that observed after the mechanical destruction of endothelium (Table 3).

Effect of miconazol

Treatment of unrubbed arteries with 10^{-6} M miconazol in the presence or absence of indomethacin did not modify the contractile response of CDRC I with respect to control (ANOVA, ns) and reversed desensitization to Ang II at doses higher than 10^{-8} M (Fig. 5). The presence of indomethacin did not modify the effect of miconazol. A significant shift to the right of CDRC II was observed, but no differences in pD_2 were found between arteries treated with miconazol and controls in CDRC I or II (Table 1).

Effect of calcium

Nifedipine (10^{-6} M) did not modify the contractile response to CDRC II in unrubbed arteries. No differences between control arteries and those treated with nifedipine were observed in contractile response or pD_2(Table 1). At the same concentration, nifedipine was able to inhibit the effect of indomethacin on desensitization to Ang II (Fig. 6). A sig-

Table 1. Values of pD_2 of cumulative dose responses curves (CDRC) to angiotensin II in rabbit aortic rings.

Treatment	*n*	CDRC I	CDRC II
E (+)	11	8.38±0.07	8.11±0.09 **
E (–)	9	8.34±0.08	8.10±0.09 **
E (+) + L-NAME	9	8.38±0.10	8.05±0.12 **
E (+) + D-NAME	9	8.24±0.10	7.83±0.13 **
E (+) + I	8	8.44±0.04	8.13±0.05 ***
E (+) + M	7	8.23±0.05	8.05±0.08 *
E (+) + I + M	7	8.25±0.05	8.04±0.04 **
E (+) + N	7	8.35±0.08	8.21±0.08 *
E (+) + I + N	7	8.29±0.07	7.94±0.17 *
E (+) + C	7	8.41±0.04	8.05±0.14 *
E (+) + I + C	7	8.19±0.09	7.62±0.15 *** †

Note: E (+) unrubbed arteries; E (–) rubbed arteries; I, indomethacin 10^{-5} M; M, miconazol 10^{-6} M; N, nifedipine 10^{-6} M; C, cromakalim 10^{-6} M. Results are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicates a statistically significant difference between CDRC I and II (paired *t* test). † $P < 0.001$ indicates a statistically significant difference between CDRC II of arteries treated with cromakalim in absence or presence of indomethacin (unpaired *t* test).

Table 2. Contractile response to NA (5×10^{-7} M) in rubbed and unrubbed rabbit aortic rings.

Aortic ring	Treatment	First (mg)	Second (mg)
E (+)	Ang II (+)	1780±432	2965±540 *
E (+)	Ang II (–)	2204±640	3287±666 *
E (–)	Ang II (+)	1962±690	3219±864 *
E (–)	Ang II (–)	2226±572	3520±715 *

Note: E (+) unrubbed arteries; E (–) rubbed arteries. Ang II (+) and Ang (–) arteries treated or not with one CDRC to Ang II between two NA stimulations (first and second). Results are expressed as mean ± SEM of 8 determinations. * $P < 0.001$ indicates a statistically significant difference between first and second noradrenaline stimulations (paired *t* test).

nificant difference in pD_2 between CDRC I and II was observed in arteries treated with nifedipine, similar to controls (Table 1).

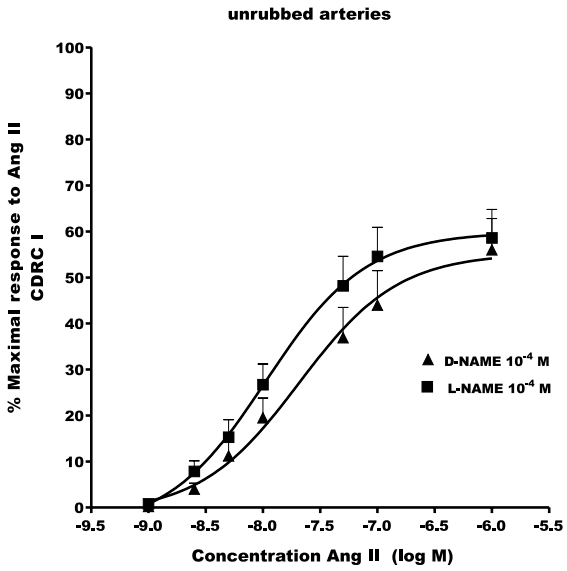
Effect of cromakalim

Cromakalim (10^{-6} M) did not modify the contractile response to CDRC II. There were no differences between control arteries and those treated with cromakalim. However, cromakalim abolished the effect of indomethacin on desensitization to Ang II, significantly increasing tachyphylaxis (Fig. 7). pD_2 of CDRC II was significantly lower in arteries treated with indomethacin plus cromakalim than pD_2 in those treated only with indomethacin (unpaired *t* test). However, there were no changes in control arteries treated only with cromakalim with respect to the pD_2 of CDRC II (Table 1).

Effect of potassium channel blockers

In unrubbed arteries, TEA (10^{-3} M), iberiotoxin (10^{-8} M), apamin (10^{-7} M), and glibenclamide (10^{-5} M) abolished Ang II-desensitization. Glibenclamide enhanced the contractile response to Ang II in the second stimulation more than the other blockers at maximal doses (Fig. 8). In rubbed arteries, TEA, apamin, and glibenclamide, but not iberiotoxin, reduced the Ang II-desensitization observed with doses lower than 5×10^{-8} M (Fig. 9), without modifications in the maximal contractile response (Table 4). In rubbed and unrubbed arteries treated with Ca^{2+} dependent K^+-channel blockers, a significant shift to the right of CDRC II was observed. However, there were no modifications regarding the magnitude of the shift in the CDRC II with respect to controls. No differences in pD_2 between CDRC I or CDRC II were found (Table 4) in rubbed and unrubbed aorta treated with glibenclamide.

Fig. 3. Cumulative dose-response curves to Ang II in rabbit aortic rings in presence of L-NAME (10^{-4} M) or D-NAME (10^{-4} M). Results are expressed as a percentage of the maximal contractile response to Ang II of the CDRC I (not significant; ANOVA and Duncan's test). Each point represents mean ± SEM of 9 experiments.



Discussion

We found that the presence of endothelium enhanced Ang II-desensitization in rabbit aortic rings. The lack of difference found in the CDRC I contractile response to Ang II in rubbed and unrubbed arteries is in agreement with a previous report stating that the endothelium does not affect the response of rabbit aortic rings to Ang II (Saye et al. 1984), but it is in contrast with the observation that Ang II responses are enhanced by disruption of the endothelium (Zhang et al. 1994). However, differences were found in the maximal contractile response between arteries treated with the NO inhibitor L-NAME and arteries with endothelium. These results mean that if Ang II releases relaxing as well as contractile factors from endothelium that its destruction would not modify smooth muscle response. On the other hand, when the synthesis of one of the relaxing factors (in this case NO) was inhibited, no counteracting action of NO was observed and the equilibrium was displaced by the release of vasoconstrictors; therefore, the maximal response observed was greater.

We did not find desensitization of the contractile response to NA in rabbit aortic rings with the present protocol. In

Fig. 4. Cumulative dose-response curves to Ang II in rabbit aortic rings in the absence (Control) and presence of indomethacin (10^{-5} M). Results are expressed as a percentage of the maximal contractile response to Ang II of the CDRC I. * $P < 0.05$, ** $P < 0.01$ indicates a statistically significant difference between control and treated tissues (ANOVA and Duncan's test). Each point represents mean ± SEM of 8 experiments.

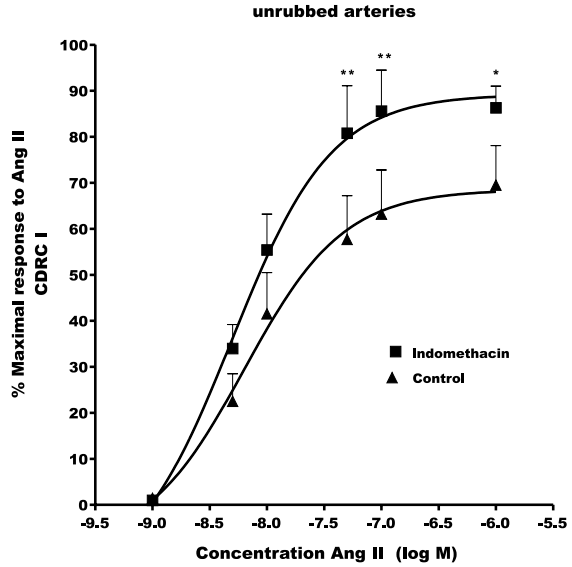


Table 3. Effect of mechanical disruption of endothelium compared with endothelium intact arteries treated with indomethacin (10^{-5} M).

Ang II Dose (M)	E (–)	E (+) + Indomethacin
10^{-9} M	2.2±1.9	0.9±0.5
5×10^{-9} M	30.7±9.3	31.3±5.2
10^{-8} M	47.8±9.1	51.2±7.8
5×10^{-8} M	70.9±10.0	75.0±10.3
10^{-7} M	71.7±10.4	79.2±8.9
10^{-6} M	91.4±9.0	86.3±4.7

Note: E (+) unrubbed arteries; E (–) rubbed arteries;. Results are expressed as a percentage (mean ± SEM of 8 determinations) of the maximal contractile response to Ang II obtained during CDRC I. Statistical analysis was performed with ANOVA and Duncan's test (not significant for all doses).

contrast, Suzuki et al. (1990) reported desensitization of the alpha 1-adrenoceptor in rabbit aorta. This disagreement may be due to the different NA incubation and recovery periods used in the experiments. The Ang II-desensitization observed in rabbit aortic rings is in agreement with a previous report by Silva et al. (1988). The presence of endothelium significantly enhanced the development of desensitization to Ang II at doses up to 10^{-8} M. However, consistent with earlier studies, desensitization still developed at low doses even in the absence of endothelium. These results suggest the existence of two mechanisms, one that is endothelium-dependent (modulates the response to the hormone), and one that involves only smooth muscle and is endothelium- independent. This is in agreement with Gruetter et al. (1987), but in contrast with Silva et al. (1988). This difference might be

Fig. 5. Cumulative dose-response curves to Ang II in rabbit aortic rings, in the absence (Control) and presence of miconazol (10^{-6} M) or miconazol plus indomethacin (10^{-5} M). Results are expressed as a percentage of the maximal contractile response to Ang II of the CDRC I. * $P < 0.01$ indicates a statistically significant difference between control and treated tissues (ANOVA and Duncan's test). Each point represents mean ± SEM of 7 experiments.

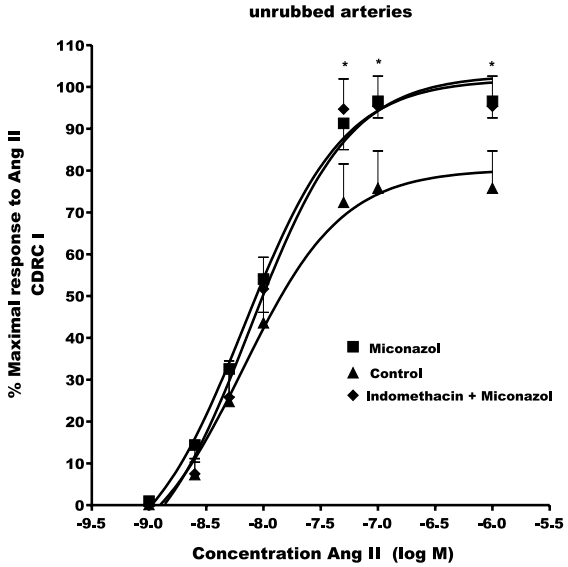


Fig. 6. Cumulative dose-response curves to Ang II in rabbit aortic rings treated with indomethacin (10^{-5} M), nifedipine (10^{-6} M) for 50 min or washing, and indomethacin plus nifedipine for 50 min of washing. There were no differences between untreated arteries (control) and those treated with nifedipine (data not shown). Results are expressed as a percentage of the maximal contractile response to Ang II of the CDRC I. * $P < 0.05$, ** $P < 0.01$ indicates a statistically significant difference between arteries treated with indomethacin and arteries with indomethacin plus nifedipine (ANOVA and Duncan's test). Each point represents mean ± SEM of 7 experiments.

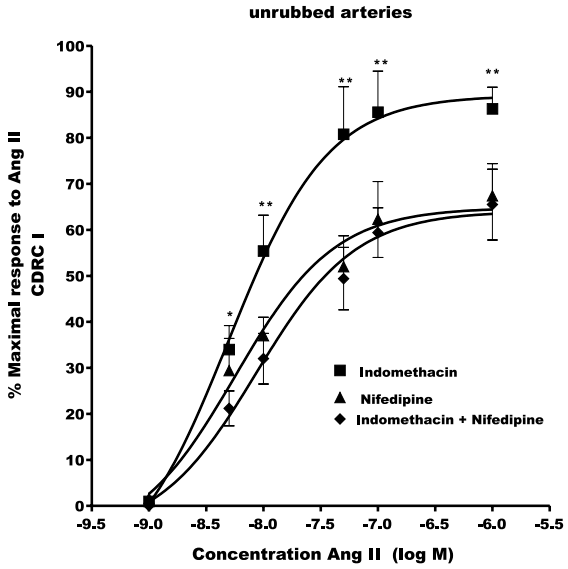
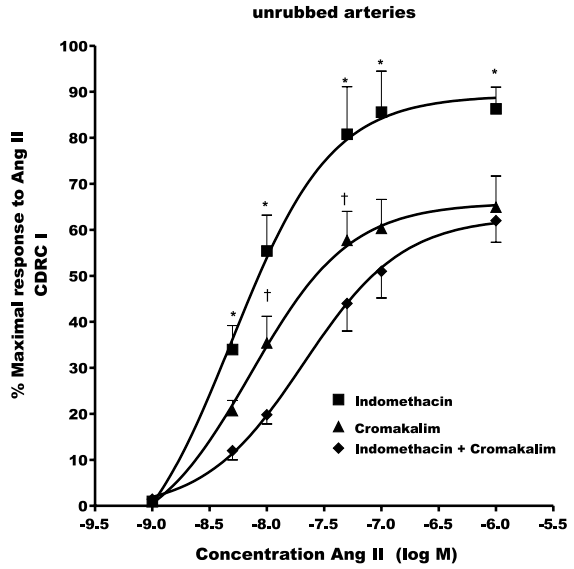


Fig. 7. Cumulative dose-response curves to Ang II in rabbit aortic rings treated with indomethacin (10^{-5} M), cromakalim (10^{-6} M) for 50 min of washing, and indomethacin plus cromakalim for 50 min of washing. There were no differences between untreated arteries (control) and those treated with cromakalim (data not shown). Results are expressed as a percentage of the maximal contractile response to Ang II of the CDRC I. * $P < 0.001$ indicates a statistically significant difference between arteries treated with indomethacin in the absence or presence of cromakalim. † $P < 0.05$ indicates a statistically significant difference between arteries treated with cromakalim in the absence or presence of indomethacin (ANOVA and Duncan's test). Each point represents mean ± SEM of 7 experiments.



due to the different protocols used because these authors induced Ang II-tachyphylaxis with one dose, which elicited the maximal contractile response, while in our study we performed two CDRC experiments. The significant shift to the right of CDRC II in rubbed arteries and the significant differences in pD_2 of the CDRC II in unrubbed arteries indicates a loss of affinity. This was observed with a similar magnitude in arteries with and without endothelium; therefore, this loss of affinity seems to be endothelium-independent. However, the presence of endothelium altered the intrinsic contractile properties of the smooth muscle cells in response to the second stimulation with Ang II, since isolated rings of unrubbed, but not rubbed, aorta developed a lower level of maximal tension.

Although L-NAME increased the maximal response to Ang II, it did not modify desensitization to Ang II in the presence of endothelium with respect to controls (arteries treated with an inactive isomer). This indicates that NO released by Ang II from endothelium has no effect in this phenomenon. Our findings are not in agreement with those observed in vasopressin tachyphylaxis in rat aorta, which is NO dependent (Millette and Lamontagne 1996).

Indomethacin and miconazol, in turn, were able to improve the contractile response to the second stimulation in arteries with endothelium. Aiken et al. (1974) found that indomethacin was able to diminish Ang II tachyphylaxis in rabbit mesenteric and celiac arteries. The rabbit aorta is traditionally considered a bioassay relatively insensitive to prostacyclin (Forstermann et al. 1984; Hardhazy et al. 1984). The hydroxylated eicosatetraenoic acids 5-HETE, 12-HETE, 15-HETE, and di5,12-HETE also had no effect on rabbit aorta under basal or induced tension (Forstermann and Neufang 1984). Carroll et al. (1987) reported that 5,6-

Fig. 8. Cumulative dose-response curves to Ang II in rabbit aortic rings with endothelium ($n = 10$) treated with apamin (10^{-7} M, $n = 7$), TEA (10^{-3} M, $n = 6$), iberiotoxin (10^{-8} M, $n = 7$) or glibenclamide (10^{-5} M, $n = 5$) for 50 min of washing. * $P < 0.05$ indicates a statistically significant difference between control arteries and those treated with apamin, TEA, iberiotoxin, or glibenclamide. † $P < 0.05$ indicates a statistically significant difference between arteries treated with glibenclamide and arteries treated with the K_{Ca}^{2+} channels blockers (ANOVA and Duncan's test). Each point represents mean ± SEM.

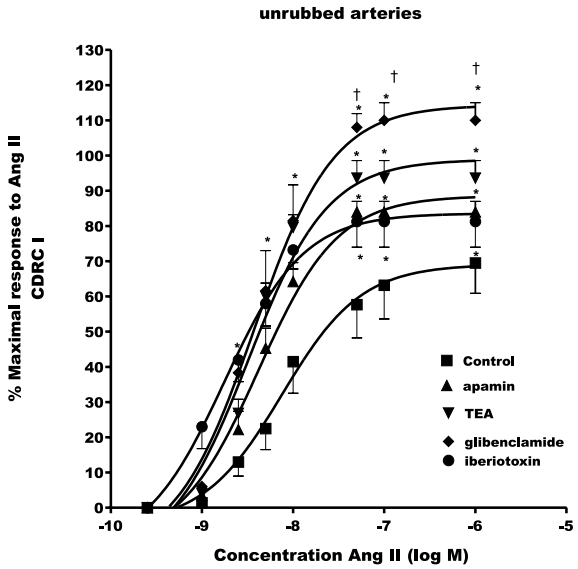


Fig. 9. Cumulative dose-response curves to Ang II in rabbit aortic rings without endothelium ($n = 8$) treated with apamin (10^{-7} M, $n = 7$), TEA (10^{-3} M, $n = 6$), iberiotoxin (10^{-8} M, $n = 7$), or

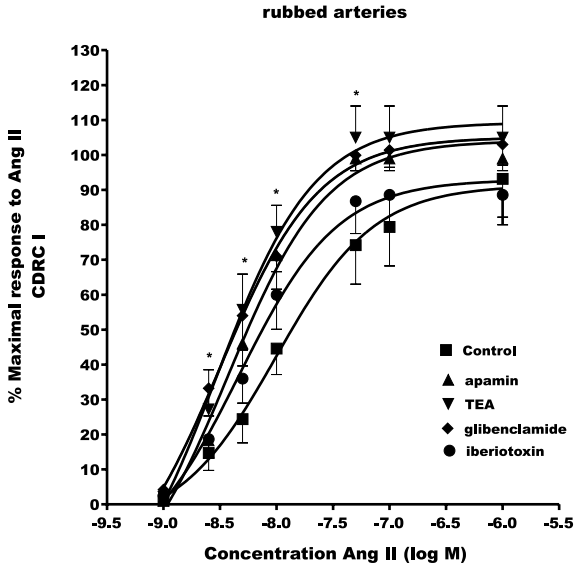


Table 4. Values of pD_2 and maximal response of cumulative dose responses curves (CDRC) to angiotensin II in rabbit aortic rings treated with different potassium channel blockers.

		CDRC I		CDRC II	
Treatment	*n*	pD2	MR (mg)	pD2	MR (mg)
E (+)	10	8.37±0.06	3967±424	8.09±0.10 **	3334±444 *
E (–)	8	8.28±0.08	4186±413	8.05±0.09 ***	4104±641
E (+) + A	7	8.59±0.03	3368±733	8.37±0.05 ***	2830±737
E (–) + A	7	8.54±0.04	2630±663	8.26±0.05 **	2681±717
E (+) + TEA	6	8.60±0.04	3469±819	8.42±0.04 ***	3412±961
E (–) + TEA	6	8.58±0.06	2309±881	8.29±0.04 ***	2234±899
E (+) + Ib	7	8.53±0.09	4142±997	8.33±0.11 **	3482±899
E (–) + Ib	7	8.60±0.11	4942±997	8.24±0.10 **	4480±1050
E (+) + G	5	8.53±0.05	3127±1051	8.35±0.11	3294±951
E (–) + G	5	8.43±0.09	3230±1018	8.28±0.11	3074±1163

Note: E (+) unrubbed arteries; E (–) rubbed arteries; A, apamin 10^{-7} M; TEA, tetraethylammonium 10^{-3} M; Ib, iberiotoxin 10^{-8} M; G, glibenclamide 10^{-5} M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicates a statistically significant difference between CDRC I and II (paired *t* test). Results are expressed as mean ± S.E.M.

epoxyeicosatrienoic acid (5,6-EET), a cytochrome P_{450} epoxygenase product, reduced the vascular resistance in perfused rat tail artery. Furthermore, 5,6-EET requires conversion by cyclooxygenase for expression of its vasoactivity (Carroll et al. 1990). Therefore, the metabolite released from endothelium by Ang II involved in the mechanism of desensitization is probably a cyclooxygenase-cytochrome P_{450} dependent product. In our work we did not find an effect of indomethacin on the contractile response to the first stimulation with Ang II, which is in agreement with Gruetter et al. (1988). This indicates that there is no basal release of cyclooxygenase products in the normal aorta, unlike pathological circumstances such as hypertension when a counterregulatory action is necessary (Dellipizzi et al. 1997). Chataigneau et al. (1999) found that endothelial denudation does not affect VSM membrane potential, indicating that basal production of cyclooxygenase products does not exert any modifying role on the membrane potential of VSMC. According to the literature (Griendling et al. 1997), when Ang II binds to its receptors in VSM it initiates a biphasic response activating phospholipase C and later on phospholipase D and phospholipase A_2. Free fatty acids (among them arachidonic acid) are released that are converted to a variety of cyclooxygenase, lipoxygenase, and cytochrome P_{450} epoxygenase products. If we suppose that Ang II has a similar mechanism of action when it binds to endothelial receptors (Pueyo and Michel 1997), some effects of the endothelium products released by Ang II on the vascular response would be evident only after some time has passed after the first stimulation with Ang II. This would explain its role in tachyphylaxis, according to our observations.

However, in arteries with endothelium treated with indomethacin or miconazol there was also a shift of CDRC II to the right that indicates a loss of affinity, even though desensitization was lower. This data, together with that mentioned above about the similar diminution of CDRC II affinity between arteries with and without endothelium, supports the view that the loss of hormone-receptor affinity is related to endothelium- independent desensitization, as stated by Kuttan and Sim (1993). On the other hand, Ullian and Linas (1989) found that the recovery $t_{1/2}$ of the number of receptors to Ang II in rat VSMC is 15 min; that is to say that after 50 min of washing there would be the same number of superficial receptors, thus, receptor decrease could not account for the Ang II-desensitization. Some experimental evidence indicates that a correlation is not always found between the number of hormonal receptors and reactivity. Benedetti and Linas (1987) demonstrated that the decrease to Ang II reactivity in the early phase of renovascular hypertension (Goldblatt) is associated with an increase in the number of binding sites. However, Kai et al. (1996) found downregulation of protein $G_{\alpha q}/G_{\alpha 11}$ levels after prolonged exposure to Ang II in intact VSMC. Nevertheless, after short-term exposure Ang II showed homologous desensitization of the IP_3 response to the subsequent stimulation, but no downregulation of $AT_{1A}R$ or $G_{\alpha q}/G_{\alpha 11}$. Thus, endothelium-independent desensitization may be attributed to receptor uncoupling.

In studies with cromakalim and nifedipine, the effect of indomethacin on desensitization was abolished. Cromakalim activates ATP-sensitive potassium channels, inducing hyperpolarization of the membrane, and nifedipine is a voltage-sensitive calcium-channel blocker. The mechanism of reduction in the response to reduced membrane potential is widely reported to be a consequence of the closure of smooth muscle voltage-sensitive channels, a reduction in Ca^{2+} influx, and subsequently a loss of vascular tone (Quilley et al. 1997). A steep relationship exists between membrane potential and Ca^{2+} influx; depolarization or hyperpolarization of as little as 3 mV can increase or decrease Ca^{2+} influx, respectively, by up to 2-fold (Nelson and Quayle 1995). Recent works (Li et al. 1997; Campbell et al. 1996) have demonstrated that vasodilator factors derived from the endothelium regulate K^+ channels in coronary VSM, producing opening and hyperpolarization. Ang II products of cyclooxygenase-cytochrome P_{450} are released that could increase Ca^{2+} dependent-potassium channel activity, which may explain the inhibition of cromakalim and nifedipine on the decrease in Ang II-tachyphylaxis by indomethacin. The result would be a lower availability of

intracellular calcium when a second stimulation by the hormone is produced.

In arteries treated with indomethacin plus cromakalim, tachyphylaxis was significantly greater than in controls. The amplified vasorelaxant effect of the K^+ channel opener could reflect increased activity of the voltage-gated Ca^{2+} channels, such that hyperpolarization, which promotes closure of these channels and reduces intracellular calcium, elicits greater vasorelaxant responses.

We also investigated the hypothesis that Ca^{2+}-dependent K^+ channels are involved in Ang II-desensitization. The K^+ channels blockers that were investigated improved the CDRC II contractile responses to Ang II in unrubbed arteries. In endothelium-denuded arteries, TEA, apamin, and glibenclamide, but not iberiotoxin, enhanced the CDRC II contractile responses to Ang II at doses lower than 5×10^{-8} M without modifications in the maximal tissue responses. Nevertheless, there was a shift to the right in arteries treated with TEA, apamin, and iberiotoxin, but not with glibenclamide. In a previous work, Cook (1989) found in rabbit aortic rings that the presence of TEA, but not apamin, produced a concentration-dependent increase in the responses to all concentrations of Ang II, irrespective of whether the endothelium was intact, and the CDRC was shifted to the left. Our protocol differed in that the CDRC was not performed in presence of the channel blocker and therefore we did not find potentiation of the Ang II-contractile response. The fact that glibenclamide prevented the loss of affinity observed in all the cases studied (including the controls) suggests that K_{ATP}-sensitive channels are involved in endothelium-independent desensitization, which is related to a shift to the right of CDRC II. The greater recovery of intrinsic activity in unrubbed arteries observed with glibenclamide compared with other potassium channel blockers is due to this effect in the VSM, independent of the presence of endothelium. With regard to Ca^{2+} dependent K^+-channels blockers, we observed that iberiotoxin was the only one that was able to improve the contractile response to CDRC II in unrubbed, but not rubbed, arteries. This allows us to conclude that endothelium-dependent desensitization is produced by a cyclooxygenase-cytochrome P_{450} product released by Ang II that increases the activity of a maxi-K^+ channel (Silva et al. 1999), whereas the effect of TEA and apamin would be unspecific.

The importance of the renin-angiotensin system in hypertension has been largely documented; many works describe the immediate effects of Ang II in vessels. In a previous work we pointed out the state in which the vessels remain after its action (Peral de Bruno et al. 1992). Hormone endothelium-dependent desensitization may play an important role in the regulation of the response to Ang II, which may be modified during endothelial dysfunction. This situation leads to hypertension through a possible imbalance in endothelium-dependent factors. There is evidence that hypertension results in a compensatory increase in the activity of K^+ channels, and possibly increased synthesis and release of the putative hyperpolarizing factors. Further studies are necessary to establish the identity of the cyclooxygenase-cytochrome P_{450} product involved in enothelium-dependent Ang II-tachyphylaxis, and to determine whether its effect is direct or through a second messenger.

Acknowledgements

The authors are indebted to Dr. Alberto Nasjletti (New York Medical College, Valhalla, N.Y., U.S.A.) for his helpful discussion. This work was supported by grants from the Consejo de Investigaciones Científicas y Técnicas de la República Argentina (P.I.P. 5008), the Consejo de Investigaciones de la Provincia de Tucumán and the Consejo de Investigaciones de la Universidad Nacional de Tucumán.

References

Aiken, J.W. 1974. Effects of prostaglandin synthesis inhibitors on angiotensin tachyphylaxis in the isolated coeliac and mesenteric arteries of the rabbit. Pol. J. Pharmacol. Pharm. **26**: 217–227.

Benedetti, R.G., and Linas, S.L. 1987. Mechanism of decreased vascular response to angiotensin II in renal vascular hypertension. Kidney Int. **31**: 906–912.

Blatz, A.L., and Magleby, K.L. 1986. Single apamin-blocked Ca^{++}-activated K^+- channels of small conductance in cultured rat skeletal muscle. Nature (London), **323**: 718–720.

Boulanger, C.M., Caputo, L., and Levy, B.I. 1995. Endothelial AT_1-mediated release of nitric oxide decreases angiotensin II contractions in rat carotid artery. Hypertension, **26**: 752–757.

Bouvier, M., Nantel, F., Valiquette, M., Moffet, S., and Mouillac, B. 1995. Le récepteur β2-adrénergique. Un modèle d'étude des mécanismes moléculaires de la désensibilisation. Méd. Sci. **11**: 819–827.

Campbell, W.B., Gebremedhin, D., Pratt, P.F., and Harder, D.R. 1996. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. Circ. Res. **78**: 415–423.

Carroll, M.A., Schwartzman, M., Capdevila, J., Falck, J.R., and McGiff, J.C. 1987. Vasoactivity of arachidonic acid epoxides. Eur. J. Pharmacol. **138**: 281–283.

Carroll, M.A., García, M.P., Falck, J.R., and McGiff, J.C. 1990. 5,6-epoxyeicosatrienoic acid, a novel arachidonate metabolite. Mechanism of vasoactivity in the rat. Circ. Res. **67**: 1082–1088.

Chataigneau, T., Feletou, M., Huang, P.L., Fishman, M.C., Duhault, J., and Vanhoutte, P.M. 1999. Acethylcholine-induced relaxation in blood vessels from endothelial nitric oxide synthase knockout mice. Br. J. Pharmacol. **126**: 219–226.

Chen, L.H., McNeill, J.R., Wilson, T.W., and Gopalakrishnan, V. 1995. Heterogeneity in vascular smooth muscle responsiveness to angiotensin II: role of endothelin. Hypertension, **26**: 83–88.

Cook, N.S. 1989. Effect of some potassium channel blockers on contractile responses of the rabbit aorta. J. Cardiovasc. Pharmacol. **13**: 299–306.

Dellipizzi, A., Pucci, M.L., Nosny, A.Y., Deseyn, K., and Nasjletti, A. 1997. Contribution of constrictor prostanoids to the calcium-dependent basal tone in the aorta from rat with aortic coarctation-induced hypertension: relationship to nitric oxide. J. Pharmacol. Exptl. Ther. **283**: 75–81.

Fasciolo, J.C., and Binia, A. 1981. Angiotensin I, II and III tachyphylaxis in the mesenteric vascular circuit of the rat. Hypertension, **3**: II166–II170.

Forstermann, U., and Neufang, B. 1984. The endothelium-dependent relaxation of rabbit aorta: effects of antioxidants and hydroxylated eicosatetraenoic acids. Br. J. Pharmacol. **82**: 765–767.

Forstermann, U., Hertting, G., and Neufang, B. 1984. The importance of endogenous prostaglandins other than prostacyclin, for the modulation of contractility of some rabbit blood vessels. Br. J. Pharmacol. **81**: 623–630.

Griendling, K.K., Ushio-Fukai, M., Lasegue, B., and Alexander, R.W. 1997. Angiotensin II signaling in vascular smooth muscle. Hypertension, **29**: 366–373.

Gruetter, C.A., Ryan, E.T., and Schoepp, D.D. 1987. Endothelium enhances tachyphylaxis to angiotensin II and III in rat aorta. Eur. J. Pharmacol. **143**: 139–142.

Gruetter, C.A., Ryan, E.T., Lenke, S.M., Bailly, D.A., Fox, M.K., and Schoepp, D.D. 1988. Endothelium-dependent modulation of angiotensin II-induced contraction in blood vessels. Eur. J. Pharmacol. **146**: 85–95.

Hardhazy, P., Malomvolgyi, B., and Magyar, K. 1984. Differential contractile responsiveness of isolated rabbit arteries from different vascular beds to cyclooxygenase inhibitors and PGI_2. Eur. J. Pharmacol. **98**: 323–330.

Hausdorff, W.P., Caron, M.G., and Lefkowitz, R.J. 1990. Turning off the signal: desensitization of β-adrenergic receptor function. FASEB J. **4**: 2881–2889.

Kai, H., Fukui, T., Lassegue, B., Shah, A., Minieri, C.A., and Griendling, K.K. 1996. Prolonged exposure to agonists results in a reduction in the levels of the G_q/G_{11} α subunits in cultured vascular smooth muscle cells. Mol. Pharmacol. **49**: 96–104.

Khairallah, P.A., Page, I.H., Bumpus, F.M., and Turker, R.K. 1966. Angiotensin tachyphylaxis and its reversal. Circ. Res. **19**: 247–254.

Kuttan, S.C., and Sim, M.K. 1993. Angiotensin II-induced tachyphylaxis in aortas of normo- and hypertensive rats: changes in receptor affinity. Eur. J. Pharmacol. **232**: 173–180.

Li, Q., Zhang, J., Pfaffendorf, M., and Van Zwieten, P.A. 1995. Comparative effects of angiotensin II and its degradation products angiotensin III and angiotensin IV in rat aorta. Br. J. Pharmacol. **116**: 2963–2970.

Li, P.L., Zou, A.P., and Campbell, W.B. 1997. Regulation of potassium channels in coronary arterial smooth muscle by endothelium-derived vasodilators. Hypertension, **29**: 262–267.

Lin, L., and Nasjletti, A. 1991. Role of endothelium-derived prostanoid in angiotensin-induced vasoconstriction. Hypertension, **18**: 158–164.

Lin, L., Balazy, M., Pagano, P.J., and Nasjletti, A. 1994. Expression of prostaglandin H_2-mediated mechanism of vascular contraction in hypertensive rats. Circ. Res. **74**: 197–205.

Millette, E., and Lamontagne, D. 1996. Endothelium-dependent and NO-mediated desensitization to vasopressin in rat aorta. Br. J. Pharmacol. **119**: 899–904.

Nelson, M.T., and Quayle, J.M. 1995. Physiological role and properties of potassium channels in arterial smooth muscle. Am. J. Physiol. **268**: C799–C822.

Peral de Bruno, M., Coviello, A., Meyer, M., and Forssmann, W.G. 1992. Relaxation of non-contracted smooth muscle by atrial natriuretic peptide. Clin. Exper. Hypertens., Part A:. **14**: 1125–1139.

Pfister, S.L., Spitzbarth, N., Nithipatikom, K., Edgemond, W.S., Falck, J.R., and Campbell, W.B. 1998. Identification of the 11,14,15- and 11,12,15-trihydroxyeicosatrienoic acids as endothelium-derived relaxing factors of rabbit aorta. J. Biol. Chem. **273**: 30 879 – 30 887.

Pueyo, M.E., and Michel, J.B. 1997. Angiotensin II receptors in endothelial cells. Gen. Pharmacol. **29**: 691–696.

Quayle, J.M., Bonev, A.D., Brayden, J.E., and Nelson, M.T. 1995. Pharmacology of ATP-sensitive K^+ currents in smooth muscle cells from rabbit mesenteric artery. Am. J. Physiol. **269**: C1112–C1118.

Quilley, J., Fulton, D., and McGiff, J.C. 1997. Hyperpolarizing factors. Biochem. Pharmacol. **54**: 1059–1070.

Sadoshima, J., Akaike, N., Tomoike, H., Kanaide, H., and Nakamura, M. 1988. Ca^{++}-activated K^+-channels in cultured smooth muscle cells of rat aortic media. Am. J. Physiol. **255**: H410–H418.

Saye, J.A., Singer, H.A., and Peach, M.J. 1984. Role of endothelium in conversion of angiotensin I to angiotensin II in rabbit aorta. Hypertension, **6**: 216–221.

Silva, E.G., Ferreira, A.T., Paiva, A.C.M., and Paiva, T.B. 1988. Angiotensin tachyphylaxis in normal and everted rings of rabbit aorta. Eur. J. Pharmacol. **153**: 185–190.

Silva, E.G., Frediani-Neto, E., Ferreira, A.T., Paiva, A.C.M., and Paiva, T.B. 1994. Role of Ca^{++}- dependent K^+ channels in the membrane potential and contractility of aorta from spontaneously hypertensive rats. Br. J. Pharmacol. **113**: 1022–1028.

Silva, B.A., Nouailhetas, V.L.A., and Aboulafia, J. 1999. Desensitization to Ang II in guinea pig ileum depends on membrane repolarization: role of maxi-K^+ channels. Am. J. Physiol. **277**: C739–C745.

Suzuki, E., Tsujimoto, G., and Hashimoto, K. 1990. Different desensitization mechanisms of two alpha 1-adrenoceptor subtypes in the contraction of rabbit aorta. Br. J. Clin. Pharmacol. **30**: 121–124.

Takizawa, H., Dellipizzi, A.M., and Nasjletti, A. 1998. Prostaglandin I_2 contributes to the vasodepressor effects of baicalein in hypertensive rats. Hypertension, **31**: 866–871.

Ullian, M.E., and Linas, S.L. 1989. Role of receptor cycling in the regulation of angiotensin II surface receptor number and angiotensin II uptake in rat vascular smooth muscle cells. J. Clin. Invest. **84**: 840–846.

Webb, M.L., Dickinson, K.E.J., Delaney, C.L., Kiu, E.C.K., Serafino, R., Cohen, R.B., Monshizadegan, H., and Moreland, S. 1992. The endothelin receptor antagonist, BQ-123, inhibits angiotensin II-induced contractions in rabbit aorta. Biochim. Biophys. Res. Comm. **185**: 887–892.

Zhang, J., Van Meel, J.C.A., Pfaffendorf, M., Zhang, J.S., and Van Zwieten, P.A. 1994. Endothelium-dependent, nitric oxide-mediated inhibition of angiotensin II-induced contractions in rabbit aorta. Eur. J. Pharmacol. **262**: 247–253.

Zhang, J., Pfaffendorf, M., Zhang, J.S., and Van Zwieten, P.A. 1995. Influence of the vascular endothelium on angiotensin II-induced contractions in rabbit renal artery. Fund. Clin. Pharmacol. **9**: 25–29.