Role of Membrane Potential and Expression of Endothelial Factors in Restenosis After Angioplasty in SHR

Janaina P. Dina, Teresa Feres, Antonio C.M. Paiva, Therezinha B. Paiva

Abstract—We examined the roles played by impaired K\textsuperscript{+} channels, diminished nitric oxide (NO) production, endothelin release, and smooth muscle membrane potential in the increased restenosis observed in spontaneously hypertensive rat (SHR) carotid arteries after angioplasty. The SHR carotid was found to be less polarized than that of normotensive Wistar rats (NWR), and it was further depolarized by the \( \alpha_2 \) agonist UK 14,304. This response was blocked by iberiotoxin, indicating that calcium-dependent K\textsuperscript{+} channels operate normally in the SHR carotid. Acetylcholine caused a hyperpolarization that was significantly smaller in SHR than in NWR carotids, indicating a deficient release of NO in the SHR. After angioplasty, SHR and NWR vessels were depolarized, returning to baseline after 10 days. In the SHR but not in the NWR the contralateral carotid was also depolarized, and this was prevented by the endothelin A/B receptor antagonist bosentan. After angioplasty, endothelin-1 plasma levels increased in both SHR and NWR, but the increase was significantly more prolonged in SHR. We found that the more pronounced restenosis observed in the SHR carotid after angioplasty is not due to impairment of calcium-dependent K\textsuperscript{+} channels but is related to the relatively depolarized vascular smooth muscles, involving endothelin release caused by reduced NO levels in that strain. (Hypertension. 2004;43:1-5.)

Key Words: rats, spontaneously hypertensive ■ angioplasty ■ carotid arteries ■ muscle smooth, vascular ■ membranes ■ endothelin

The vascular endothelium plays a role in blood flow control through the release of relaxant and contractile factors. Thus, to prevent overactivation of contractile responses, it releases relaxant factors such as endothelium-derived hyperpolarizing factor, prostanoids, and nitric oxide (NO). The endothelium also prevents the development of vascular lesions by inhibiting platelet aggregation, leukocyte adhesion, and proliferation of vascular smooth muscle cells. This protective role is altered in pathological conditions such as arterial hypertension, which is characterized by cell permeability changes, leukocyte adhesion, and smooth muscle cell proliferation. Also, the repair mechanism designed to restore the vessel wall after damage from different causes frequently escapes self-limiting control, resulting in lumen narrowing caused by smooth muscle cell proliferation. These processes are the precursors of atherosclerotic plaque formation, which leads to increased vascular resistance and to restenosis.

Procedures such as atherectomy performed by balloon injury (angioplasty) widen the lumen but cause extensive endothelium destruction, leaving the vascular wall without protection. This injury stimulates medial smooth muscle cell proliferation and migration to the deendothelized surface to form a neointima. Neointima proliferation and subsequent vascular restenosis are believed to be the main events in the initiation of the atherosclerosis that limits the long-term efficacy of percutaneous transluminal coronary angioplasty.

It is well accepted that reduction in nitric oxide activity accounts for endothelial dysfunction, since NO donors reduce intimal hyperplasia and oxidative stress aggravates this process. Moreover, there is evidence that endothelin is an important factor in neointima formation after vascular injury. Elevated endothelin plasma levels were found in patients after angioplasty.

In spontaneously hypertensive rat (SHR) carotid arteries, Clowes and Clowes attributed increased neointima formation after balloon injury to the hypertensive state. Jandeleit-Dahm et al reported similar results in renovascular hypertensive rats but proposed that the restenosis could be due to the increased concentration of angiotensin-converting enzyme found in these animals. Furthermore, effective antihypertensive dosages of verapamil did not prevent neointima formation in SHR, indicating that the hypertensive state is not responsible for the restenosis after arterial injury in SHR. Other genetic factors besides the hypertensive state are probably responsible for the increased restenosis in this strain. In addition, SHR presented a more pronounced proliferative response to balloon injury than renovascular hypertensive or normotensive rats. Since resting membrane potential measurements showed that the SHR carotid smooth muscle cells are depolarized in comparison to those of either renovascular hypertensive or normotensive rats, the authors concluded that the depolarized state rather

Received August 26, 2003; first decision October 3, 2003; revision accepted October 24, 2003.
From the Department of Biophysics, Escola Paulista de Medicina, Federal University of São Paulo, São Paulo, Brazil.
Correspondence to Therezinha B. Paiva, MD, PhD, Department of Biophysics, Federal University of São Paulo, Rua Botucatu, 862, 04023-032 São Paulo, SP, Brazil. E-mail tbrpaiva@biofis.epm.br © 2003 American Heart Association, Inc.
Hypertension is available at http://www.hypertensionaha.org DOI: 10.1161/01.HYP.0000105300.74809.4F
than the hypertension could account for the increased proliferative response in SHR.

In previous articles, we reported that SHR mesenteric artery smooth muscles are less polarized than those of normotensive Wistar rats (NWR). This was attributed to different alterations, such as impairment of the activity of K⁺-channels coupled to α₁-adrenoceptors or diminished NO production in the SHR.

In the present work, we investigated the role of K⁺-channels and NO production in the smooth muscle cell membrane potentials of carotid arteries of SHR and NWR. We also followed the effect of angioplasty on the smooth muscle cell membrane potentials of injured and contralateral vessels and on the endothelin plasma levels in these animals.

**Methods**

Chemicals

Acetylcholine chloride and iberiotoxin were purchased from Sigma Chemical Co, Bosentan was from Actelion, and UK 14,304 (5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinazolinamine) was from Research Biochemicals International. The other chemicals were products of the highest analytical grade from Merck Darmstadt.

Animals

Experiments were carried out with the use of male Okamoto-Aoki SHR and NWR rats, 20 to 30 weeks old, weighing 250 to 300 g.

Injury Model

The animals were anesthetized by intraperitoneal injection of 20 mg/kg body wt thiophenbutal (Abbott) and 30 mg/kg body wt chloral hydrate, and the left external and common carotid arteries were exposed. With the aid of a micrometer and magnifying glass, the external diameter of the common carotid artery was measured in situ and used for calibrating balloon expansion. A balloon catheter was passed through the external carotid artery and advanced into the aorta, inflated with saline, and pulled back to distend the common carotid artery to 1.2 times its external diameter. The latter procedure was repeated twice, after which the external carotid artery was ligated and the incision was sutured. The animals were anesthetized by intraperitoneal injection of 20 mg/kg body wt thiophenbutal (Abbott) and 30 mg/kg body wt chloral hydrate, and the left external and common carotid arteries were exposed. With the aid of a micrometer and magnifying glass, the external diameter of the common carotid artery was measured in situ and used for calibrating balloon expansion. A balloon catheter was passed through the external carotid artery and advanced into the aorta, inflated with saline, and pulled back to distend the common carotid artery to 1.2 times its external diameter. The latter procedure was repeated twice, after which the external carotid artery was ligated and the incision was sutured. The extent of neointima formation obtained by this procedure,

Membrane Potential Measurements

Carotid artery rings were placed in a 2-mL perfusion chamber, superfused at a rate of 3 mL/min with Krebs-Henseleit solution maintained at 37°C, and bubbled with a 5% CO₂–95% O₂ mixture. After an equilibration period of 40 minutes under an optimal resting tension of 1.0 g, membrane potentials were recorded by means of microelectrodes constructed by pulling capillaries in a horizontal puller (Narishige, model PN3). The pipettes were filled with 2 mol/L KCl and had tip resistance of 20:40 mol/L. The electrodes were mounted in Ag/AgCl half-cells on a micromanipulator (Leitz) and connected to an electrometer (WP Instruments, model FD 223). The signals were recorded in a potentiometric chart recorder (EBC, model RB102). Rings of 1-cm length were cut from the carotid artery, placed in a 2-mL perfusion chamber, and superfused at a rate of 3 mL/min with Krebs solution of the following composition (in mmol/L): NaCl 137; NaHCO₃ 5.9; KHCO₃ 5.9; CaCl₂ 2.3; MgCl₂ 1.2; glucose 11.8. The solution was bubbled with 5% CO₂–95% O₂ gas mixture and maintained at 37°C, pH 7.4. The impalements were made directly in the smooth muscle cells from the adventitial side. The successful implantation of the electrode was evidenced by a sharp drop in voltage on entry into a cell, a stable potential (±3 mV) for at least 1 minute after impalement, and a sharp return to zero on exit. The time of contact of the drugs with the preparations before the impalements was 10 minutes.

Plasma Endothelin-1 Measurements

Blood samples, collected in glass tubes containing 50 μL Liquemine (Roche), were centrifuged at 2000g for 10 minutes at 4°C, and 2 mL plasma was acidified with 0.25 mL 2 mol/L HCl and centrifuged at 10000g for 5 minutes, then loaded onto a 500 mg Amprep C2 column (Amersham) previously equilibrated with methanol followed by water. The column was washed with 5 mL water, 0.1% trifluoroacetic acid, and 80% HPLC grade methanol in water. The eluate was dried under nitrogen, the pellet was reconstituted in 250 μL assay buffer, and 100-μL duplicates were taken for quantitative enzyme-linked immunosorbent assay (ELISA) with the use of a Biotrack kit (Amersham) according to the manufacturer's instructions.

Statistics

Statistical analysis was carried out by 1-way ANOVA followed by the Newman-Keuls test in the case of pairwise comparisons between groups. Data are expressed as mean±SEM. When the data consisted of repeated observations at successive time points, ANOVA for repeated measurements was applied to determine differences between groups. When more than one impalement was made on the same ring from the same rat, the measurements were averaged and considered as n=1. Differences were considered significant at a value of P<0.05.

**Results**

Effects of UK 14,304 and Iberiotoxin on Membrane Potentials

SHR carotid smooth muscle membrane potentials were significantly less negative (depolarized) than those of NWR (Figure 1). To determine the role of calcium-dependent K⁺-channels in this relatively depolarized state of the SHR, we examined the effect of UK14,304, an opener of these channels, on the membrane potential of both hypertensive and normotensive animals.

Figure 1 shows that the SHR carotid membrane potential was increased by UK 14,304, and this response was totally blocked by the addition of iberiotoxin, an inhibitor of calcium-dependent K⁺-channels. This indicates that these channels operate normally in the SHR carotid.

The role of EDRF was tested in SHR and NWR carotid rings by means of the responses to acetylcholine. As shown in...
Figure 2, the hyperpolarization induced by this agonist was significantly smaller in SHR (−12.2 mV) than in NWR (−18.6 mV), indicating a deficient release of NO in the SHR carotids.

Evolution of Membrane Potentials of Injured and Contralateral Carotids After Angioplasty

Figure 3 shows the membrane potentials measured at different times after angioplasty in the injured and in the contralateral carotids. In both strains, a significant depolarization was present in the injured vessels 2 days after surgery, and the membrane potentials remained at the same depolarized level until the 7th day, returning to normal values by the 10th day. In the contralateral vessels, however, a different behavior was observed in the two strains. In the SHR, the contralateral vessels showed a depolarization and time course of recovery similar to those of the injured vessels (Figure 3B). This parallelism between the ipsilateral and contralateral carotid membrane potentials after angioplasty was not observed in the NWR (Figure 3A).

The responses of ipsilateral and contralateral arteries to UK 14,304, as well as their inhibition by IBTX, measured 5 days after angioplasty, were not significantly different from those measured before the procedure was performed (not shown).

Plasma Endothelin Concentration Before and After Angioplasty

The finding that injured and contralateral carotids from SHR undergo similar depolarization after surgery suggests the participation of a humoral factor released from the injured carotid into the circulation. In view of reports of endothelin release after mechanical lesion of endothelial cells, measurements of this peptide’s plasma levels before and after angioplasty were performed.

Endothelin-1 plasma levels in the SHR and the NWR were not significantly different, and a marked increase in the plasma concentration of this peptide was observed in both strains by the fourth day after surgery (Figure 4). By the 7th day, the endothelin level had returned to normal in the NWR but remained high in the SHR, returning to normal by the 15th day.

To determine whether endothelin release was responsible for the parallelism between the depolarizations of injured and contralateral carotids in the SHR, membrane potentials were measured in these arteries before and 2 days after angioplasty, in the presence and in the absence of the endothelin A/B receptor antagonist bosentan. Figure 5 shows that this endothelin antagonist prevented the depolarization of both carotids, indicating that endothelin release may be responsible for the depolarization observed in the contralateral vessel.
Endothelin release between the endotelin-1 plasma levels of SHR or NWR, in agreement with previous reports. Thus, endothelin release might explain the depolarization. After angioplasty, a depolarization of the injured vessel was observed in both the SHR and the NWR, which, however, lasted longer in the SHR. The endothelin plasma levels were also increased in the two strains but were also more persistent in the SHR. This could be due to the reduced NO concentration in this strain, since NO is known to impair endothelin production. The more pronounced endothelin release into the circulation after angioplasty in the SHR was probably responsible for the parallel depolarizations of injured and contralateral arteries in this strain. Although the contralateral vessels were depolarized to the same extent as the injured carotids, they did not develop restenosis, since the endothelium was intact.

In conclusion, we have found that the more pronounced restenosis observed in the SHR carotid after angioplasty is not due to impairment of calcium-dependent K⁺ channels but is related to the relatively depolarized vascular smooth muscles, involving endothelin release caused by reduced NO levels in that strain.

**Perspectives**

Our results demonstrate that the increased restenosis observed in SHR after angioplasty is favored by vascular smooth muscle cell depolarization caused by increased plasma endothelin concentration as the result of lower NO production. K⁺Ca channels are not impaired in this animal model of hypertension. Neointima formation after angioplasty may be reduced by procedures that decrease vascular smooth muscle depolarization such as endothelin blockade by specific inhibitors or by increasing the plasma NO concentration.

**Acknowledgments**

This work was supported by grants and fellowships from the São Paulo State Research Foundation (FAPESP) and by the Brazilian National Research Council (CNPq). We are grateful to Nelson Farias for some of the experimental data and to Nelson Alves Mora for competent technical assistance.

**References**


**Figure 5.** Membrane potentials measured in carotid artery rings from SHR in the absence (RMP) and presence of 3 µmol/L bosentan. For each ring obtained from individual rats (number shown in parentheses below bars), 5 to 7 cells were impaled, and averages of respective measurements were used to obtain mean±SEM. *P<0.05 vs respective RMP (Newman-Keuls test).


Role of Membrane Potential and Expression of Endothelial Factors in Restenosis After Angioplasty in SHR
Janaina P. Dina, Teresa Feres, Antonio C.M. Paiva and Therezinha B. Paiva

Hypertension. published online November 24, 2003;

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2003/11/24/01.HYP.0000105300.74809.4F.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/