ELSEVIER

European Journal of Pharmacology 394 (2000) 301–309

ejp

www.elsevier.nl/locate/ejphar

Angiotensin I-converting enzyme activity and vascular sensitivity to angiotensin I in rat injured carotid artery

Jacinthe Lemay [a], Ying Hou [a], Johanne Tremblay [b], Denis deBlois [a,*]

[a] *Department of Pharmacology, Laboratory of Pharmacology of Vascular Pathologies, Centre de Recherche du C.H.U.M., Université de Montréal, 3840 St. Urbain Street., 7-133B, Montréal, Québec Canada H2W 1T8,*

[b] *Department of Medicine, Laboratory of Cellular Biology of Hypertension, Centre de Recherche du C.H.U.M., Université de Montréal, 3840 St. Urbain Street., 7-133B, Montréal, Québec H2W 1T8, Canada*

Received 8 November 1999; received in revised form 17 January 2000; accepted 21 January 2000

Abstract

We used a vasoreactivity assay to examine the functional significance of angiotensin I-converting enzyme overexpression in smooth muscle cells after vascular injury. Rat carotid arteries isolated at days 2 to 14 after in vivo endothelial denudation were compared with the contralateral freshly denuded (control) vessels. Arterial rings were constricted ex vivo with angiotensin I in the absence or presence of the angiotensin I-converting enzyme inhibitors captopril (300 nM and 3 μM) or perindoprilate (1 nM). Angiotensin I-converting enzyme activity was determined by cleavage of the chromogenic substrate Hip-His-Leu. Angiotensin I-converting enzyme activity in injured arteries was increased (2-fold) at day 7 only after vascular injury. Contractions to angiotensin I were unaffected after injury. Inhibition by captopril and perindoprilate of angiotensin I-induced contractions was significantly less potent in injured arteries at day 7 as compared to control vessels. Mechanical removal of neointimal smooth muscle cells normalized the inhibition by captopril in injured arteries at day 7. Captopril did not affect angiotensin II-induced contractions. Thus, upregulation of angiotensin I-converting enzyme after arterial injury confers resistance to angiotensin I-converting enzyme inhibitors.

Keywords: Carotid artery, rat; Endothelial injury; Angiotensin I-converting enzyme; Angiotensin I-converting enzyme inhibitor; Vasoconstriction

1. Introduction

Angiotensin II is a stimulant of the response to vascular injury, a complex condition that involves smooth muscle cell contraction, migration and growth ultimately leading to neointimal lesion formation (Schwartz et al., 1995). Angiotensin I-converting enzyme cleaves the inactive peptide angiotensin I to generate angiotensin II. Angiotensin I-converting enzyme inhibitors were first shown to block vascular lesion formation in the rat model of carotid artery injured with a balloon catheter (Powell et al., 1989). In this model, angiotensin I-converting enzyme inhibitors reduce neointimal hyperplasia mainly by suppressing the early response to injury in the media, including smooth muscle cell proliferation and migration (Prescott et al., 1991; Timmermans et al., 1993; Fingerle et al., 1995). After balloon angioplasty, rat arteries exhibit increased angiotensin I-converting enzyme mRNA and protein expression, and accelerated enzymatic cleavage of synthetic substrate specific for angiotensin I-converting enzyme (Rakugi et al., 1994a,b; Fishel et al., 1995; Fernandez-Alfonso et al., 1997). Rat neointimal smooth muscle cells also show enhanced angiotensin I-converting enzyme expression, particularly in the luminal layers of the lesion (Fishel et al., 1995), where cell proliferation is predominant (Clowes et al., 1986). In human coronary artery plaques, immunoreactive angiotensin I-converting enzyme protein is found in macrophages (Diet et al., 1996; Ohishi et al., 1997a), and its expression is induced in plaque smooth muscle cells after balloon angioplasty (Ohishi et al., 1997b). These findings were taken as additional evidence implicating tissue angiotensin I-converting enzyme in vascular remodeling. In freshly denuded rat carotid arteries, however, angiotensin I and angiotensin II show comparable potency as vasocontrictors (Schiffers et al., 1991; Ackerman et al., 1998), suggesting that smooth muscle cell angiotensin

* Corresponding author. Tel.: +1-514-843-2919; fax: +1-514-843-2911.

E-mail address: debloisd@pharmco.umontreal.ca (D. deBlois).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00071-6

I-converting enzyme activity is not a rate-limiting step in the generation of angiotensin II in the media of this vascular bed. Suppression of neointimal formation requires higher doses of angiotensin I-converting enzyme inhibitor than for reduction of blood pressure (Rakugi et al., 1994b). In these rats, residual smooth muscle cell angiotensin I-converting enzyme activity remains greater in injured than uninjured vessels, suggesting that smooth muscle cells in denuded arteries may be relatively more resistant to angiotensin I-converting enzyme inhibitors as compared to smooth muscle cells in uninjured arteries (Rakugi et al., 1994b). To the best of our knowledge, there is no study in which angiotensin I-converting enzyme-mediated conversion of angiotensin I to angiotensin II was evaluated in injured vessels. Thus, the functional significance of angiotensin I-converting enzyme upregulation remains unclear with regard to smooth muscle cell sensitivity to angiotensin I in injured rat carotid arteries. The present study was designed to answer the following questions: (1) Are responses to angiotensin I increased in rat denuded carotid artery with upregulated angiotensin I-converting enzyme? and (2) is the efficacy of angiotensin I-converting enzyme inhibitors altered in denuded arteries with upregulated angiotensin I-converting enzyme? Arterial rings isolated at various times after endothelial denudation were examined using a vasoreactivity assay in the presence and absence of angiotensin I-converting enzyme inhibitors. Our results show that arterial angiotensin I-converting enzyme activity is not a rate-limiting step in mediating angiotensin I effects in rat denuded carotid arteries. However, the upregulation of smooth muscle cell angiotensin I-converting enzyme decreases the efficacy of angiotensin I-converting enzyme inhibitors in injured arteries.

2. Materials and methods

2.1. Surgical procedures

Endothelial denudation with a rotating nylon filament loop was used as a well-characterized model of vascular injury that results in significant neointima formation (Fingerle et al., 1990) but that is associated with minimal disruption of the media (Fingerle et al., 1990; Lindner and Reidy, 1991; Lindner et al., 1992) compared to balloon angioplasty, thus reducing the variance in vasoactive response measurements. The investigation was conducted in accordance with institutional guidelines and the recommendations of the Declaration of Helsinki and Tokyo. The left common carotid artery of male Sprague–Dawley rats (350–400 g, from Charles River Laboratories, St-Constant, Canada) was denuded of endothelium as described previously (Fingerle et al., 1990), in accordance with institutional guidelines, in animals anesthetized with a single intramuscular injection of ketamine (50 mg/kg; MTC Pharmaceuticals, Canada), xylazine (5 mg/kg; Bayer, Canada) and acepromazine (2.5 mg/kg; Ayerst Laboratories, Canada). Five minutes prior to sacrifice at days 2, 7 or 14 after injury, they were anesthetized with enflurane (Abbott, Canada) and given an intravenous injection of Evan's Blue solution (60 mg/kg; ICN Biomedicals, Canada) to stain arterial segments that are chronically denuded of endothelium. The animals were then sacrificed with saline perfusion through the abdominal aorta; the left (injured) and right (uninjured) common carotid arteries were immediately isolated, placed in ice-cold saline solution, and cleaned free of adventitial adipose and connective tissue. In all control uninjured carotid arteries studied, the endothelium was mechanically removed by intimal rubbing using a 20-gauge needle inserted in the vessels cut into arterial rings. A subset of injured arteries isolated at day 7 was subjected to a similar procedure of intimal rubbing to examine the role of superficial neointimal smooth muscle cells in regulating vascular angiotensin I-converting enzyme expression and responses to angiotensin I.

2.2. Angiotensin I-converting enzyme activity measurement

Arterial angiotensin I-converting enzyme activity was determined by fluorometric assay measuring His-Leu generation from Hippuril-His-Leu as described by Cushman and Cheung (Cushman et al., 1971). Immediately after isolation, whole common carotid arteries cleaned free of adventitial adipose and connective tissue, were weighed after endothelium removal as described above, snap frozen in liquid nitrogen, pulverized with a mortar and pestle, transferred (15 mg wet tissue equivalent/ml) in assay buffer (300 mM NaCl, 100 mM KH_2PO_4, pH 7.5), and sonicated for 40 s on ice. Aliquots of the crude undiluted arterial homogenates (100 μl) were added to an equal volume of assay buffer and incubated for 10 min at 37°C in the presence of Hippuril-His-Leu (10 mM; Sigma, MO, USA). Preliminary studies confirmed that under those conditions His-Leu generation was linear over 1 h and directly proportional to the volume of homogenate assayed (data not shown). To measure background fluorescence, paired samples were incubated in the presence of captopril (100 μM). The reaction was stopped by the addition of NaOH (0.28 M). His-Leu was tagged with 0.1% *o*-phtalaldehyde (Sigma) and quantitated fluorometrically at an excitation wavelength of 363 nm and emission wavelength of 486 nm without further sample dilution. The His-Leu standard curve ranged from 0.0625 to 1 nM. Arterial angiotensin I-converting enzyme activity was expressed as nanomoles of His-Leu generated per minute per milligram of protein. In a separate experiment, the concentration-dependent inhibition of angiotensin I-converting enzyme by captopril was examined. Freshly denuded and injured vessels (day 7) were isolated from eight rats, pooled for processing as described above and assayed for Hippuril-His-Leu cleavage in the presence of captopril (0.0003–1000 μM).

2.3. Vasoreactivity

The contractility assay was chosen because smooth muscle cell contraction is an early event following receptor stimulation thus allowing the estimation of biologically relevant angiotensin II levels in the vascular tissue exposed to angiotensin I. Freshly-isolated vessels without adventitial adipose and connective tissue or endothelium were cut into 3-mm-long rings and placed at 37°C in organ chambers filled with oxygenated Krebs solution containing in mM: dextrose, 11; NaCl, 117.5; $MgSO_4$, 1.18; KH_2PO_4, 1.2; $NaHCO_3$, 25; KCl, 4.7; $CaCl_2$, 2.5. Isometric contractions were measured with the use of isometric force transducers (Harvard Apparatus Canada), a digitalized data acquisition system (Model MP100, Biopac System, Harvard) and a computer (LC630, Apple, Canada). The arterial rings were placed in organ chambers under 1 g of tension and allowed to stabilize for 1 h before control stimulation with phenylephrine (100 nM; Sigma). Preliminary studies in our laboratory have shown these conditions to be optimal for evaluation of vascular function. Briefly, tissues were stimulated twice with KCl (70 mM) under each of the following levels of basal tension: 0.5, 1.0, 1.5 and 2.0 g. We found that 1.0 and 1.5 g of tension yielded the highest contractile responses to KCl. Since there was no difference between these two tensions, the lowest value (1.0 g) was selected for the present studies. Previous studies showed that optimal tension does not change significantly in rat carotid arteries within 2 weeks after endothelial denudation (Douglas et al., 1994). To confirm the absence of endothelium, acetylcholine (1 μM; ICN Biomedicals, Canada) was added to phenylephrine-precontracted vessels to verify the absence of endothelial-dependent vasorelaxation by acetylcholine. After a 1-h recuperation period, the rings were submitted to cumulative concentrations of angiotensin I or angiotensin II (10^{-10} to 10^{-5} M; Sigma), with or without captopril (0.3–3 μM; Sigma) or perindoprilate (1 nM; gift from Servier) which were added 20 min prior to stimulation with the angiotensin peptides. One hour later, tissues were stimulated with KCl (70 mM; ICN Biomedicals). Arterial rings were stimulated only once to avoid angiotensin I- or angiotensin II-induced tachyphylaxis. Vasoactive responses were expressed as g of contraction and arterial sensitivity as pD_2 (minus the log of agonist concentration which elicits the half-maximal response).

2.4. Histological studies

The number of smooth muscle cell nuclei present in the media and neointima was evaluated histologically in a randomly-selected subset of arterial rings obtained from vessels used for vasoactive studies. Arterial segments were obtained from the middle portion of the freshly denuded and chronically denuded. To quantify smooth muscle cell proliferation at the time of vascular tissue isolation, a randomly selected subset of rats received a single intraperitoneal injection of bromodeoxyuridine (15 mg/kg; Boehringer Mannheim, Canada) 1 h before death at days 2, 7 or 14 after denudation. This procedure results in specific labelling of nuclei undergoing DNA synthesis in vivo (Zeymer et al., 1992). Nuclear bromodeoxyuridine immunoreactivity in smooth muscle cells was detected in 4% paraformaldehyde-fixed, paraffin-embedded arterial tissue sections (5 μm), using a primary G-type immunoglobulin (IgG) anti-bromodeoxyuridine antibody (Boehringer) and a secondary sheep anti-mouse IgG antibody (Vector Laboratories, Canada), as described previously (deBlois et al., 1996). All slides were also stained with hematoxyline (Sigma). The total number of smooth muscle cell nuclei and bromodeoxyuridine-positive smooth muscle cell nuclei per cross section were evaluated by manually counting the nuclei on the slides using 400 × magnification on a Zeiss Axioskop microscope (Zeiss, Canada). Smooth muscle cell proliferation was expressed as the percentage of bromodeoxyuridine-positive smooth muscle cell nuclei over total smooth muscle cell nuclei. The results were expressed separately for the media and neointima.

2.5. Statistical analysis

The data are expressed as mean ± S.E.M.. The results were analyzed using the Student's *t*-test for paired samples comparing freshly denuded and injured arteries from the same animal. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Validation of the model of response to injury

The filament loop endothelial denudation model of vascular injury was used instead of balloon angioplasty

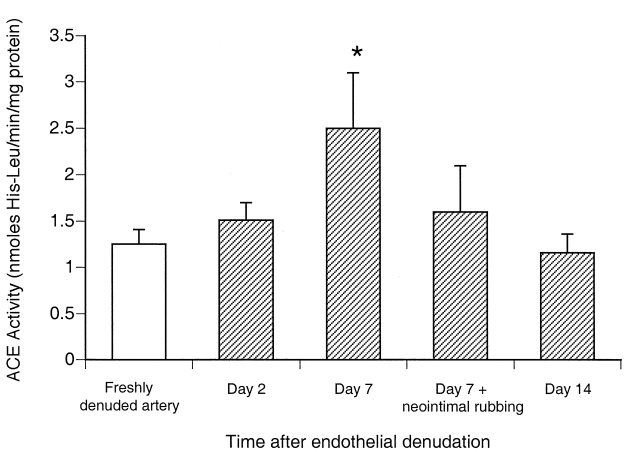

Fig. 1. Induction of arterial angiotensin I-converting enzyme (ACE) activity in rat common carotid arteries at day 2, 7 and 14 after endothelial denudation; $n = 8–9$. Data are presented as mean ± S.E.M. $^*P < 0.05$ vs. the contralateral freshly denuded control carotid artery.

Table 1
Contractions (g) induced by KCl and phenylephrine in rings of rat carotid arteries isolated at various times after endothelial denudation
Control arteries were freshly denuded carotid arteries isolated from the same rat as injured arteries. The Student's *t*-test for paired samples revealed no significant differences in contractile responses between freshly denuded arteries and injured arteries.

Time after injury	In vitro treatment	*n*	KCl (70 mM)		Phenylephrine (100 nM)	
			Injured artery	Freshly denuded artery	Injured artery	Freshly denuded artery
2 days						
	Control	13	0.88 ± 0.09	0.86 ± 0.13	0.84 ± 0.15	0.79 ± 0.08
	Captopril (0.3 μM)	13	0.73 ± 0.09	0.73 ± 0.09	0.84 ± 0.08	0.71 ± 0.13
7 days						
	Control	18	0.77 ± 0.07	0.88 ± 0.14	0.65 ± 0.08	0.52 ± 0.04
	Captopril (0.3 μM)	9	0.84 ± 0.10	0.74 ± 0.10	0.67 ± 0.08	0.48 ± 0.07
	Captopril (3 μM)	9	0.75 ± 0.11	0.83 ± 0.09	0.61 ± 0.08	0.54 ± 0.05
	Perindoprilate (1 nM)	12	1.09 ± 0.09	1.11 ± 0.10	0.70 ± 0.08	0.83 ± 0.06
	Neointimal rubbing + captopril (0.3 μM)	12	0.93 ± 0.11	1.01 ± 0.06	0.72 ± 0.11	0.64 ± 0.09
14 days						
	Control	12	0.50 ± 0.20	0.62 ± 0.26	0.54 ± 0.09	0.56 ± 0.06
	Captopril (0.3 μM)	12	0.86 ± 0.09	0.63 ± 0.26	0.68 ± 0.22	0.42 ± 0.13

because more reproducible vascular reactivity studies can be achieved since this procedure does not damage the media. Thus, smooth muscle cell proliferation and angiotensin I-converting enzyme activity measurements were used to validate the response to injury. Cross-sections of freshly denuded arteries showed 300 ± 10 smooth muscle cell nuclei in the media and no smooth muscle cells in the intima ($n = 31$). In response to endothelial denudation with a filament loop, smooth muscle cell nuclei number did not change significantly in the media at all the time points studied (not shown) but increased significantly in the neointima (e.g. at day 7: 351 ± 36 and day 14: 670 ± 47; $n = 10–31$). As shown in Fig. 1 smooth muscle angiotensin I-converting enzyme activity was enhanced transiently at day 7 post-denudation, but not at days 2 and 14. Angiotensin I-converting enzyme activity seen in injured vessels at day 7 was reduced by mechanically removing the superficial smooth muscle cell layers of the neointima (neointimal rubbing). This procedure at day 7 also resulted in a 45% reduction in the total number of neointimal smooth muscle cells (194 ± 64 nuclei left remaining; $n = 12$), and a 95% reduction in the percentage of proliferating smooth muscle cells (1.0 ± 1.0% vs. 20.3 ± 1.3%; $n = 31$). Thus, arteries injured with a nylon filament loop behaved as reported previously for balloon-injured vessels in terms of smooth muscle cell proliferation and accumulation. Furthermore, this procedure led to transiently increased angiotensin I-converting enzyme activity in the arterial wall.

3.2. Vasoactive studies

In each rat, the freshly denuded right carotid artery was used as an internal control for the injured left carotid artery. Comparing these vessels revealed no difference in contractility to KCl (70 mM; Table 1) to phenylephrine

Table 2
Arterial sensitivity (pD_2) and maximal contractile responses (E_{max}; g) to angiotensin II in rings of rat carotid arteries isolated at different times after endothelial denudation
pD_2 is the negative logarithm of the angiotensin II concentration eliciting the half-maximal contractile response. Control arteries were freshly denuded carotid arteries isolated from the same rat as injured arteries. The Student's *t*-test for the paired samples revealed no significant differences in arterial sensitivity or contractile responses between freshly denuded arteries and injured arteries.

Time after injury	In vitro treatment	*n*	pD_2		E_{max}	
			Injured artery	Freshly denuded artery	Injured artery	Freshly denuded artery
2 days						
	Control	18	8.59 ± 0.08	8.78 ± 0.09	1.04 ± 0.08	0.90 ± 0.08
7 days						
	Control	9	8.58 ± 0.09	8.51 ± 0.07	1.03 ± 0.18	0.94 ± 0.10
	Captopril (0.3 μM)	9	8.53 ± 0.05	8.71 ± 0.19	0.89 ± 0.19	0.99 ± 0.09
14 days						
	Control	10	8.21 ± 0.12	8.33 ± 0.09	0.59 ± 0.10	0.68 ± 0.08

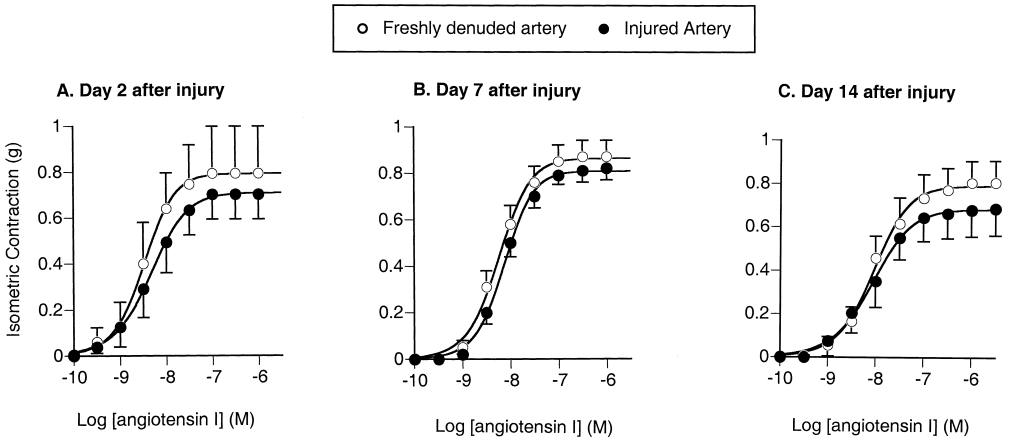

Fig. 2. Contractile responses to angiotensin I in freshly denuded control arteries vs. arteries isolated at days 2, 7 and 14 after endothelial denudation (A, B and C respectively); $n = 7–18$. Data are presented as mean ± S.E.M.

(100 nM; Table 1) or to angiotensin II (Table 2) after injury. The contractile responses to angiotensin I were similar between freshly denuded and injured arteries at all the times studied (Fig. 2). Moreover, arterial sensitivity (expressed as pD_2) for angiotensin I was not increased after injury (Table 3). Thus, there was no evidence of increased arterial responsiveness to angiotensin I in rat injured carotid arteries with angiotensin I-converting enzyme upregulation. To further analyze the functional significance of smooth muscle cell angiotensin I-converting enzyme upregulation after arterial injury, vasoactive responses were evaluated in the presence of angiotensin I-converting enzyme inhibitors. Incubation with captopril (300 nM) reduced sensitivity to angiotensin I in all vessels, as evidenced by the rightward shift in the concentration–response curves at days 7 and 14 and to a lesser extent at day 2 (Fig. 3A–C, Table 3). Under these conditions, however, angiotensin I-induced contractions were stronger in injured arteries at day 7 compared to freshly denuded arteries. In the presence of captopril, injured arteries isolated at day 7 showed greater arterial sensitivity (pD_2) for angiotensin I compared with freshly denuded vessels (Table 3). Similar results showing increased angiotensin I effects in injured vs. freshly denuded arteries were observed in vessels incubated in the presence of perindoprilate (1 nM) at day 7 after injury (Fig. 3D, Table 3). Incubation with a larger dose of captopril (3 μM) also resulted in greater arterial sensitivity to angiotensin I in injured arteries vs. paired freshly denuded arteries (Table 3). In contrast, angiotensin II-induced contractions were not different in freshly denuded vs. injured arteries incubated with captopril (0.3 μM) at day 7 after injury (Table 2). Since the increased levels of angiotensin I-converting enzyme activity seen in injured arteries at day 7 are

Table 3
Arterial sensitivity (pD_2) to angiotensin I in rings of rat carotid arteries isolated at various times after endothelial denudation
pD_2 is the negative logarithm of the angiotensin I concentration eliciting the half-maximal contractile response. Control vessels were freshly denuded carotid arteries isolated from the same rat as injured arteries.

Time after injury	In vitro treatment	*n*	Injured artery	Freshly denuded artery
2 days				
	Control	13	8.50 ± 0.14	8.70 ± 0.12
	Captopril (0.3 μM)	13	8.41 ± 0.18	8.40 ± 0.16
7 days				
	Control	8	8.43 ± 0.08	8.21 ± 0.07
	Captopril (0.3 μM)	9	7.13 ± 0.10[a]	6.94 ± 0.15
	Captopril (3 μM)	9	6.50 ± 0.15[a]	6.36 ± 0.16
	Perindoprilate (1 nM)	12	7.86 ± 0.18[a]	7.51 ± 0.17
	Neointimal rubbing + captopril (0.3 μM)	12	6.71 ± 0.15	6.75 ± 0.08
14 days				
	Control	12	8.08 ± 0.15	8.04 ± 0.19
	Captopril (0.3 μM)	12	7.23 ± 0.17	7.28 ± 0.17

[a] Significant difference ($P < 0.05$) between freshly denuded arteries and injured arteries, as determined by the Student's *t*-test for paired samples.

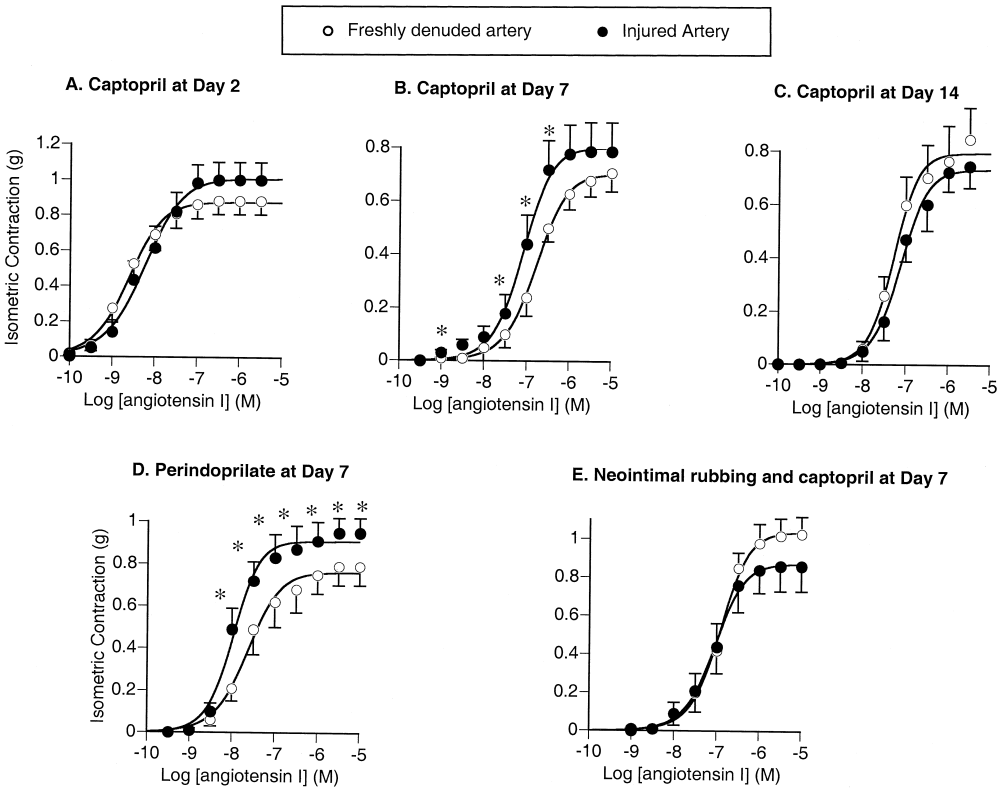

Fig. 3. Contractile responses to angiotensin I in the presence of captopril (0.3 μM) in freshly denuded control arteries vs. arteries isolated at days 2, 7 and 14 after endothelial denudation (A, B and C respectively); $n = 9–18$. (D) Effect of perindoprilate (1 nM) on the contractile responses to angiotensin I at day 7 after injury; $n = 12$. (E) Effect of intimal rubbing with a metal rod on angiotensin I-induced contractions in the presence of captopril (0.3 μM) in injured arteries at day 7; $n = 12$. Data are presented as mean ± S.E.M. $^{*}P < 0.05$ vs. the corresponding response in the contralateral freshly denuded control carotid artery.

reduced by rubbing off the superficial cell layers in the neointima, we investigated the results of this intervention on the regulation of angiotensin I contractile effects in injured arteries. As shown in Fig. 3E, injured arteries incubated with captopril following neointimal rubbing at day 7 exhibited contractile responses to angiotensin I similar to those of paired freshly denuded vessels. Incubation of freshly denuded vessels in the presence of Evan's Blue (5 mg/ml) did not affect the contractile responses to angiotensin I (E_{max}: 0.78 ± 0.12 g; pD_2: 8.30 ± 0.15; $n = 4$) as compared to vessels with out Evan's Blue (E_{max}: 0.82 ± 0.09 g; pD_2: 8.40 ± 0.20; $n = 4$). As shown in Fig. 4 angiotensin I-converting enzyme activity in tissue homogenates was inhibited by captopril in a concentration-dependent fashion. However, residual angiotensin I-converting enzyme activity was greater in injured arteries as compared to the freshly denuded artery for captopril concentration below 3 μM. In summary, incubation with angiotensin I-converting enzyme inhibitors had a weaker suppressing effect on angiotensin I-induced contractions in injured vs. freshly denuded control carotid arteries, and this correlated with greater levels of residual angiotensin I-converting enzyme activity.

Angiotensin I-converting enzyme is a key enzyme in inactivation of the vasoactive peptide bradykinin (Erdos, 1990). The application of a maximal concentration of bradykinin (1 μM) did not affect vascular tone in absence of endothelium in resting or phenylephrine-precontracted

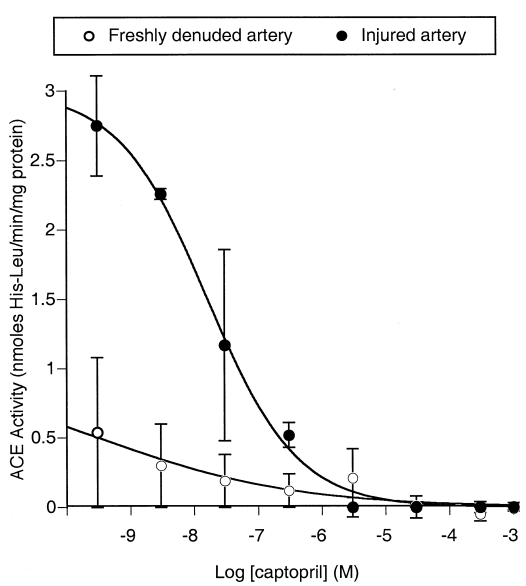

Fig. 4. Angiotensin I-converting enzyme (ACE) activity in the presence of increasing concentrations of captopril in homogenates of freshly denuded and injured arteries isolated at day 7 after in vivo endothelial denudation. Data are presented as mean ± S.E.M. of triplicate measurements obtained with a pool of vessels from eight rats.

rings of freshly denuded arteries or arteries isolated at 2 to 14 days after injury (data not shown).

4. Discussion

The upregulation of smooth muscle cell angiotensin I-converting enzyme after balloon catheterization in rat (Schwartz et al., 1995) and human (Ohishi et al., 1997b) arteries raises the possibility that angiotensin II generation is increased locally, resulting in enhanced angiotensin II-dependent vascular stenosis. Thus, our aim was to determine whether vascular angiotensin I-converting enzyme upregulation after endothelial injury enhances vascular sensitivity to angiotensin I under basal conditions and in the presence of angiotensin I-converting enzyme inhibitors.

Our results show that vascular angiotensin I-converting enzyme is not increased in injured arteries at days 2 and 14 after endothelial denudation. As expected, in these injured vessels the contractile responses to angiotensin I were not different from freshly denuded arteries. At day 7, however, injured arteries showed no potentiation of angiotensin I contractile effects even though vascular angiotensin I-converting enzyme activity was increased by 2-fold. We confirmed that endothelial cells were absent in the preparations studied, as determined by in vivo Evan's Blue staining and by the absence of acetylcholine-dependent relaxation on phenylephrine-precontracted vessels. Therefore, we conclude that endothelium did not contribute to the vasoactive responses measured with angiotensin I and angiotensin II. Although we cannot rule out the possible contribution of non-smooth muscle cell to angiotensin I-converting enzyme activity in the present model, the interpretation that smooth muscle cells are the main cell type responsible for increased angiotensin I-converting enzyme activity after injury is supported by several studies (Rakugi et al., 1994a; Fishel et al., 1995; Fernandez-Alfonso et al., 1997). Control contractile responses to phenylephrine or KCl were also not different after injury, suggesting that injury did not interfere with the intrinsic contractility of the vessels. Taken together, these results indicate that upregulation of smooth muscle cell angiotensin I-converting enzyme does not increase vascular responsiveness to angiotensin I in the injured rat carotid artery. Arterial tissue efficacy in converting angiotensin I to angiotensin II is vessel- and species-specific. Upregulation of vascular angiotensin I-converting enzyme increases angiotensin I to angiotensin II conversion in the perfused isolated hindlimb preparation of two-kidney, one-clip hypertensive rats (Muller et al., 1997). Using a similar vascular preparation, the group of Inagami (Okamura et al., 1986) reported that angiotensin I-converting enzyme is not a rate-limiting step in the generation of angiotensin II in the hindlimb of normotensive rats. The relative potency of angiotensin I over angiotensin II in contractile assays is markedly greater in cerebral vs. thoracic or abdominal arteries in the rabbit, and it is 10 times greater in rat vs. rabbit aortae (Zerrouk et al., 1996). Several groups have reported comparable potencies for angiotensin I and angiotensin II in contracting freshly denuded rat carotid arteries (Schiffers et al., 1991; Ackerman et al., 1998). Accordingly our study suggests that angiotensin I-converting enzyme is not a rate limiting step for angiotensin II generation in the rat carotid artery with regards to vascular contractility. Clearly, vessel-specific levels of angiotensin I-converting enzyme activity should be taken into account when discussing the implications of angiotensin I-converting enzyme upregulation in vascular diseases.

A key finding of the present study is that injured arteries with angiotensin I-converting enzyme upregulation show increased resistance to angiotensin I-converting enzyme inhibitors in a vasoreactivity assay. In the presence of a submaximal concentration of captopril, the suppression of angiotensin I contractile effects was more pronounced in freshly denuded arteries as compared to paired injured vessels at day 7. Incubation with the structurally-distinct angiotensin I-converting enzyme inhibitor perindoprilate also revealed weaker inhibition of angiotensin I-induced contractions in injured arteries, suggesting a class effect for angiotensin I-converting enzyme inhibitors. The doses of angiotensin I-converting enzyme inhibitors tested were in the plasma concentration range found in hypertensive patients taking these medications (Duchin et al., 1982; Lees, 1990). Control dose–response curves to angiotensin II were carried out to investigate angiotensin I-converting enzyme-independent vasoconstrictions by the angiotensin II pathway in injured arteries. We found no significant difference in angiotensin II-induced contraction between control and injured arteries at days 2, 7 and 14. In contrast to angiotensin I, incubation with captopril did not affect contractility to angiotensin II. Furthermore, we observed that in the presence of a submaximal concentration of angiotensin I-converting enzyme inhibitor (< 3 μM captopril), injured arteries have greater residual angiotensin I-converting enzyme activity than freshly denuded vessels. Under these conditions angiotensin I-converting enzyme may thus become a rate-limiting step for vascular contractility, and angiotensin I-dependent contractions are greater in injured than control vessels.

It is intriguing to note that the effects of captopril (at a submaximal concentration) were somewhat less important in vessels (both injured and uninjured) isolated at day 2. This may reflect the upregulation of non-angiotensin I-converting enzyme pathways for vascular angiotensin II generation as part of a systemic response to surgical stress. Alternative pathways for angiotensin II generation, e.g. chymases, are present and upregulated with injury in monkey, dog, human but not rat vessels (Nishimura et al., 1996). The presence of a non-angiotensin I-converting enzyme, chymostatin-sensitive pathway for angiotensin II generation was recently described in freshly isolated rat carotid arteries (Inoue et al., 1999). This activity was

detectable at high concentrations of angiotensin I (> 100 nM) in the presence of complete angiotensin I-converting enzyme inhibition. This activity may thus account in part for angiotensin I-induced contractions in the presence of an angiotensin I-converting enzyme inhibitor in the present study. Whether upregulation of such a pathway contributed to the reduced efficacy of captopril in all vessels at day 2 remains to be determined. Alternatively, angiotensin-degrading enzymes may be reduced at that time. Clearly, numerous factors are likely to affect the biological activity of angiotensin I in injured vessels. It is important to note, however, that the main conclusions of the present report remain valid in the face of these considerations, i.e. increased angiotensin I-converting enzyme activity after injury was not associated with increased potency of angiotensin I but rather with reduced efficacy of angiotensin I-converting enzyme inhibitors.

It has been shown that angiotensin I-converting enzyme expression or activity is predominant in smooth muscle cells in the luminal layers of the neointima (Fishel et al., 1995). The present results show that removal of superficial, proliferating smooth muscle cells in the neointima reduced angiotensin I-converting enzyme activity and prevented resistance to captopril in injured arteries, suggesting that proliferating neointimal smooth muscle cells may influence local arterial sensitivity to angiotensin I-converting enzyme inhibitors. In rats, it was shown that higher doses of angiotensin I-converting enzyme inhibitor are needed to inhibit arterial angiotensin I-converting enzyme activity and neointima formation after balloon catheterization compared with inhibition of plasma angiotensin I-converting enzyme activity and systemic blood pressure (Rakugi et al., 1994b). Moreover, suppression of neointima formation correlates more closely with inhibition of angiotensin I-converting enzyme activity in arterial tissue than plasma (Rakugi et al., 1994b). Prescott et al. (1991) reported that angioplasty-induced medial smooth muscle cell proliferation in rat arteries is inhibited by the angiotensin II AT_1 receptor antagonist losartan but not the angiotensin I-converting enzyme inhibitor benazepril when these drugs are given at doses equipotent for blood pressure reduction. The present data suggest that smooth muscle cell angiotensin I-converting enzyme upregulation may contribute to the difficulty of reaching significant inhibition of tissue angiotensin I-converting enzyme and neointimal formation. This may provide a therapeutic advantage for angiotensin II AT_1 receptor antagonists over angiotensin I-converting enzyme inhibitors in suppressing vascular remodeling after injury.

This study was focused on the functional significance of enhanced vascular angiotensin I-converting enzyme expression with regard to the vasoactive responsiveness to angiotensin I in denuded vessels. However, some limitations of the present study need to be addressed. We used the contractile effect of angiotensin I as a surrogate marker for angiotensin I-converting enzyme-dependent generation of angiotensin II leading to receptor stimulation. This study did not address the issue of whether angiotensin I-converting enzyme is rate limiting for the downstream effects of angiotensin II on smooth muscle cell growth, as these may be different between injured and uninjured vessels. Indeed, numerous studies suggest that the smooth muscle cell growth response to angiotensin II is potentiated in the injured arterial wall (Schwartz et al., 1995). Moreover, local transfection of the angiotensin I-converting enzyme gene in vivo in non-denuded rat carotid arteries enhances angiotensin II AT_1 receptor-dependent smooth muscle cell growth locally (Morishita et al., 1994). In this model or intraluminal DNA delivery, severe arterial dilation stimulated smooth muscle cell proliferation even in vector-transfected vessels (Morishita et al., 1994). Thus, caution should be exercised in extending the present conclusions to arteries with endothelium because complex regulation of the smooth muscle cell response may result in injured vessels with endothelium (Hahn et al., 1995).

In summary, this study provided evidence that angiotensin I-converting enzyme is a necessary but not a limiting step in mediating the vasoconstrictive effects of angiotensin I in the rat denuded carotid artery. We found, however, that arterial angiotensin I-converting enzyme upregulation does not lead to increased responsiveness to angiotensin I but rather confers vascular resistance to angiotensin I-converting enzyme inhibitors. These results are potentially relevant to conditions where angiotensin II-dependent vascular stenosis is predominant.

Acknowledgements

The work was supported by the Heart and Stroke Foundation of Quebec awarded to D. deBlois. D. deBlois is a junior scholar of the the Fonds de Recherche en Santé du Québec. J. Lemay received a studentship from the Medical Research Council of Canada.

References

Ackerman, A., Fernandez-Alfonso, M.S., Sanchez de Rojas, R., Ortega, T., Paul, M., Gonzalez, C., 1998. Modulation of angiotensin-converting enzyme by nitric oxide. Br. J. Pharmacol. 124, 291–298.

Clowes, A.W., Clowes, M.M., Reidy, M.A., 1986. Kinetics of cellular proliferation after arterial injury III. Endothelial and smooth muscle growth in chronically denuded vessels. Lab. Invest. 54, 295–303.

Cushman, D.W., Cheung, H.S., 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem. Pharmacol. 20, 1637–1648.

deBlois, D., Viswanathan, M., Su, J.E., Clowes, A.W., Saavedra, J.M., Schwartz, S.M., 1996. Smooth muscle DNA replication in response to angiotensin II is regulated differently in the neointima and media at different times after balloon injury in the rat carotid artery: role of AT_1 receptor expression. Arterioscler. Thromb. Vasc. Biol. 16, 1130–1137.

Diet, F., Pratt, R.E., Berry, G.J., Momose, N., Gibbons, G.H., Dzau, V.J.,

1996. Increased accumulation of tissue ACE in human atherosclerotic coronary artery disease. Circulation 94, 2756–2767.

Douglas, S.A., Vickery-Clarck, L.M., Ohlstein, E.H., 1994. Functional evidence that balloon angioplasty results in transient nitric oxide synthase induction. Eur. J. Pharmacol. 255, 81–89.

Duchin, K.L., Singhvi, S.M., Willard, D.A., Migdalof, B.H., McKinstry, D.N., 1982. Captopril kinetics. Clin. Pharmacol. Ther. 31, 452–458.

Erdos, E.G., 1990. Angiotensin I-converting enzyme and the changes in our concepts through the years: Lewis K. Dahl memorial lecture. Hypertension 16, 363–370.

Fernandez-Alfonso, M.S., Martorana, P.A., Licka, I., Van Even, P., Trobisch, D., Scholkens, B.A., Paul, M., 1997. Early induction of angiotensin I-converting enzyme in rat carotid artery after balloon injury. Hypertension 30, 272–277.

Fingerle, J., Au, Y.P., Clowes, A.W., Reidy, M.A., 1990. Intimal lesion formation in rat carotid arteries after endothelial denudation in absence of medial injury. Arteriosclerosis 10, 1082–1087.

Fingerle, J., Muller, R.M., Kuhn, H., Pech, M., Baumgartner, H.R., 1995. Mechanism of inhibition of neointimal formation by the angiotensin-converting enzyme inhibitor cilazapril. A study in balloon catheter-injured rat carotid arteries. Arterioscler. Thromb. Vasc. Biol. 15, 1945–1950.

Fishel, R.S., Thourani, V., Eisenberg, S.J., Shai, S.-Y., Corson, M.A., Nabel, E.G., Bernstein, K.E., Berk, B.C., 1995. Fibroblast growth factor stimulates angiotensin converting enzyme expression in vascular smooth muscle cells. J. Clin. Invest. 95, 377–387.

Hahn, A.W., Schmidt, R., Kern, F., Resink, T.J., Buhler, F.R., 1995. Endothelium-modulated proliferation of medial smooth muscle cells: influence of angiotensin II and converting enzyme inhibition. Eur. Heart J. 16 (Suppl. C), 29–32.

Inoue, K., Nishimura, H., Kubota, J., Kawamura, K., 1999. Alternative angiotensin II formation in rat arteries occurs only at very high concentrations of angiotensin I. Hypertension 34, 525–530.

Lees, K.R., 1990. Clinical pharmacology of perindopril. J. Hum. Hypertens. 4 (Suppl. 4).

Lindner, V., Olson, N.E., Clowes, A.W., Reidy, M.A., 1992. Inhibition of smooth muscle cell proliferation in injured rat arteries. Interaction of heparin with basic fibroblast growth factor. J. Clin. Invest. 90, 2044–2049.

Lindner, V., Reidy, M.A., 1991. Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. Proc. Natl. Acad. Sci. U. S. A 88, 3739–3743.

Morishita, R., Gibbons, G.H., Ellison, K.E., Lee, W., Zhang, L., Yu, H., Kaneda, Y., Ogihara, T., Dzau, V.J., 1994. Evidence for direct local effect of angiotensin in vascular hypertrophy. In vivo gene transfer of angiotensin converting enzyme. J. Clin. Invest. 94, 978–984.

Muller, D.N., Bohlender, J., Hilgers, K.F., Dragun, D., Costerousse, O., Menard, J., Luft, F.C., 1997. Vascular angiotensin-converting enzyme expression regulates local angiotensin II. Hypertension 29, 98–104.

Nishimura, H., Hoffmann, S., Baltatu, O., Sugimura, K., Ganten, D., Urata, H., 1996. Angiotensin I converting enzyme and chymase in cardiovascular tissues. [Review] [63 refs] Kidney Int. (Supplement 55), S18–S23.

Ohishi, M., Ueda, M., Rakugi, H., Naruko, T., Kojima, A., Okamura, A., Higaki, J., Ogihara, T., 1997a. Enhanced expression of angiotensin-converting enzyme is associated with progression of coronary atherosclerosis in humans. J. Hypertens. 15, 1295–1302.

Ohishi, M., Ueda, M., Rakugi, H., Okamura, A., Naruko, T., Becker, A.E., Hiwada, K., Kamitani, A., Kamide, K., Higaki, J., Ogihara, T., 1997b. Upregulation of angiotensin-converting enzyme during the healing process after injury at the site of percutaneous transluminal coronary angioplasty in humans. Circulation 96, 3328–3337.

Okamura, T., Miyazaki, M., Inagami, T., Toda, N., 1986. Vascular renin–angiotensin system in two-kidney, one clip hypertensive rats. Hypertension 8, 560–565.

Powell, J.S., Clozel, J., Muller, R., Kuhn, H., Hefti, F., Hosang, M., Baumgartner, H., 1989. Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. Science 245, 186–188.

Prescott, M., Webb, R., Reidy, M.A., 1991. Angiotensin-converting enzyme inhibitors vs. angiotensin II, AT1 receptor antagonist: effects on smooth muscle cell migration and proliferation after balloon catheter injury. Am. J. Pathol. 139, 1291–1302.

Rakugi, H., Kim, D.K., Krieger, J.E., Wang, D.S., Dzau, V.J., Pratt, R.E., 1994a. Induction of angiotensin-converting enzyme in the neointima after vascular injury. Possible role in restenosis. J. Clin. Invest. 93, 339–346.

Rakugi, H., Wang, D.S., Dzau, V.J., Pratt, R.E., 1994b. Potential importance of tissue angiotensin-converting enzyme inhibition in preventing neointima formation. Circulation 90, 449–455.

Schiffers, P.M., Struyker-Boudier, H.A., De Mey, J.G., 1991. Effects of angiotensin II and angiotensin converting enzyme inhibitors on contractile and growth responses in isolated carotid arteries of the rat. Basic Res. Cardiol. 86 (Suppl. 1), 83–89.

Schwartz, S.M., deBlois, D., O'Brien, E.R., 1995. The intima. Soil for athrosclerosis and restenosis. Circ. Res. 77, 445–465.

Timmermans, P.B.M.W.M., Wong, P.C., Chiu, A.T., Herblin, W.F., Benfield, P., Carini, D.J., Lee, R.J., Wexler, R.R., Saye, J.A.M., Smith, R.D., 1993. Angiotensin II receptors and angiotensin II receptor antagonists. Pharmacol. Rev. 45, 205–251.

Zerrouk, A., Auguet, M., Delaflotte, S., Chabrier, P.E., 1996. Effects of angiotensin I and angiotensin II in blood vessels: greater influence of converting enzyme activity in the rabbit basilar artery. Naunyn Schmiedeberg's Arch. Pharmacol. 354, 466–473.

Zeymer, U., Fishbein, M.C., Forrester, J.S., Cercek, B., 1992. Proliferating cell nuclear antigen immunohistochemistry in rat aorta after balloon denudation: comparison with thymidine and bromodeoxyuridine labeling. Am. J. Pathol. 141, 685–690.