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Kubo-Inoue, Mayuko, Kensuke Egashira, Makoto Usui, Masao Takemoto, Kisho Ohtani, Makoto Katoh, Hiroaki Shimokawa, and Akira Takeshita. Long-term inhibition of nitric oxide synthesis increases arterial thrombogenecity in rat carotid artery. *Am J Physiol Heart Circ Physiol* 282: H1478–H1484, 2002. First published November 29, 2001; 10.1152/ajpheart.00739.2001.—Reduced activity of endothelial nitric oxide (NO) may be involved in thrombus formation on atherosclerotic plaques, a major cause of acute coronary syndrome. However, mechanisms of such increase in arterial thrombogenecity have not been fully understood. We previously reported that long-term inhibition of NO synthesis by administration of N^G-nitro-L-arginine methyl ester (L-NAME) causes hypertension and activates vascular tissue angiotensin-converting enzyme (ACE) activity. We used this model to investigate the mechanism by which long-term impairment of NO activity increases arterial thrombogenecity. We observed cyclic flow variations (CFVs), a reliable marker of platelet thrombi, after the production of stenosis of the carotid artery in rats treated with L-NAME for 4 wk. The thrombin antagonist argatroban suppressed the CFVs. The CFVs were detected in rats receiving L-NAME plus hydralazine but not in rats receiving L-NAME plus an ACE inhibitor (imidapril). Treatment with the ACE inhibitor imidapril, but not with hydralazine, prevented L-NAME-induced increases in carotid arterial ACE activity and attenuated tissue factor expression. These results suggest that long-term inhibition of endothelial NO synthesis may increase arterial thrombogenecity at least in part through angiotensin II-induced induction of tissue factor and the resultant thrombin generation. These data provide a new insight as to how endothelial NO exhibits antithrombogenic properties of the endothelium.

thrombosis; angiotensin-converting enzyme; tissue factor; thrombin

OCCLUSIVE OR NONOCCLUSIVE thrombus formation on atherosclerotic plaque is believed to be of prime importance in the cause of acute coronary syndrome as well as the progression of atherosclerosis. Such thrombus formation is triggered by endothelial dysfunction, by exposure of plaque components or local mediators in the subendothelial layers to blood components after endothelial disruption (plaque rupture), or by systemic blood factors (17, 25). Thus any interventions that may reduce the risk of thrombus formation might be expected to improve patient outcomes. The antithrombotic effects of nitric oxide (NO) have been studied mainly in in vitro conditions or in in vivo conditions with acute blockade of NO activity. However, the mechanisms by which long-term impairment of NO activity can increase arterial thrombogenesis are not fully understood.

Atherosclerosis has been demonstrated to be associated with endothelial dysfunction, which leads to the abnormal production of NO. Endothelial NO has been shown to regulate vascular tone and to inhibit platelet aggregation, thrombus formation, leukocyte adhesion, and vascular proliferation (3, 6, 7, 10, 11, 18). Recently, we and others have reported that chronic inhibition of NO synthesis by administration of N^G-nitro-L-arginine methyl ester (L-NAME) induces early inflammation [monocyte infiltration, monocyte chemoattractant protein-1 (MCP-1) expression, and nuclear factor (NF)-κB activation] and late cardiovascular remodeling through increases in angiotensin-converting enzyme (ACE) and angiotensin II activities in rats (14–16, 26, 28, 30). Although angiotensin II is not a direct platelet agonist, it is known that angiotensin II may increase arterial thrombogenesis by activation of coagulation factors such as tissue factor (TF) (4, 21) or by inhibition of fibrinolytic factors via activation of plasminogen activator inhibitor-1 (PAI-1) or inhibition of plasminogen activator (13, 21, 31) via angiotensin II type 1 receptor stimulation. We have reported that ACE inhibition with imidapril prevented gene expression, protein production, and activity of PAI-1 in this model (13). However, it is unclear whether arterial thrombogenicity is increased through angiotensin II activity in the rat model of chronic inhibition of NO synthesis.

In the present study, we used this model to investigate whether arterial thrombogenesis is increased in the carotid artery of rats with chronic inhibition of NO synthesis. We show here that cyclic flow variations (CFVs), a reliable marker of platelet thrombus forma-

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tion (9), develop after stenosis-related damage in the rat model. We further show that ACE inhibition prevented the occurrence of CFVs through a decrease in TF activity. Our present data suggest that angiotensin II-induced expression of TF and the resultant production of thrombin in the arterial wall may mediate arterial thrombogenesis in this model.

METHODS

Animal model of chronic inhibition of NO synthesis. The study protocol was reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and the experiments were conducted according to the Guidelines for Animal Experiments of Kyushu University Faculty of Medicine. A part of this study was performed at the Kyushu University Station for Collaborative Research.

Five groups of 20-wk-old male Wistar-Kyoto rats were studied. The control group received untreated chow and drinking water. The second group (acute L) received L-NAME intravenously (10 mg/kg) after the operation. The third group (chronic L) received L-NAME in its drinking water (1 mg/ml) (22, 26). The fourth group (L+Hyd) received L-NAME and hydralazine (0.12 mg/ml) in its drinking water. The fifth group (L+Imi) received L-NAME and the ACE inhibitor imidapril (0.2 mg/ml) in its drinking water. We (13, 14, 26) have previously reported that imidapril and hydralazine at the dose used normalized the L-NAME-induced increase in systolic blood pressure.

On *day 28* of treatment, systolic blood pressure and heart rate (by the tail-cuff method) were measured. The rats were then euthanized for physiological, morphometric, and biochemical analyses.

Surgical preparation, introduction of stenosis, and histopathological analysis. Rats were anesthetized with pentobarbital (50 mg/kg ip), and the right common carotid artery was exposed. An ultrasonic transit-time flow probe (IRB1381, Transonic Systems) was placed immediately distal to the bifurcation of the common carotid artery, and its blood flow was measured with a flowmeter (T106, Transonic System).

After the carotid blood flow (CBF) was recorded and was stable for at least 30 min, stenosis was introduced with a 4-0 silk suture. The external surface of the common carotid artery from 5 to 7 mm distal to the bifurcation was stenosed (12). A piece of stainless steel needle was placed alongside the exposed segment of the carotid artery, and a suture was firmly placed around both the needle and the vessel; the needle was then removed, leaving a narrowed lumen. A needle 0.4 mm in diameter was used to introduce 75% stenosis. Before and after the stenosis was introduced, CBF was monitored and recorded at least for 30 min, and the occurrence of CFVs was determined. In some rats in the control group, more severe 90% stenosis was introduced. A needle 0.5 mm in diameter was used to introduce such severe stenosis. CFVs were evaluated by their frequency (cycles per 30 min) and by severity [average of the 3 lowest blood flow values (nadir) relative to CBF before production of stenosis].

If CFVs were provoked and continued for at least 30 min in rats of the chronic L group, either vehicle (5% glucose) or the thrombin antagonist argatroban at 1 mg/kg was administered intravenously to determine the role of thrombin in the pathogenesis of CFVs. To determine whether the thrombogenic effect of L-NAME was related to inhibition of NO synthesis, L-arginine at 70 mg/kg was administered intravenously. We (28) previously reported that this dose of L-arginine restored NO-generating capacity in rats with chronic L-NAME treatment (28). If CFVs progressed to irreversible zero CBF, the rats were euthanized, and the carotid arteries were harvested for histological analysis to demonstrate histopathological evidence of thrombus formation. If the animal did not develop CFVs or if the animal developed CFVs in the absence of irreversible zero flow, the rat was euthanized for histopathological examination of the carotid arteries.

For histopathological analysis, the excised carotid arteries were fixed for 3 days with 6% formaldehyde, dehydrated, embedded in paraffin, and cut into 5-μm-thick sections. Sections were mounted on slides and stained with hematoxylin-eosin solution. The whole area of all the histopathological sections was scanned using a light Nikon Microphot-FXA. To evaluate the thickening of the carotid artery wall, short-axis images of the arteries were studied.

Platelet aggregation and prothrombin time determination. To determine whether L-NAME affected platelet function, the response to collagen was evaluated ex vivo in a different set of animals. Five milliliters of blood were drawn into a syringe containing 0.5 ml of 3.8% sodium citrate and centrifuged at 120 *g* for 20 min at room temperature to obtain platelet-rich plasma (PRP). PRP was recentrifuged at 1,000 *g* for 5 min to obtain platelet-poor plasma (PPP). Platelet aggregation was measured turbidimetrically on a Chronolog aggregometer and recorded on a linear recorder. The aggregometer was calibrated by the use of PRP, and the test was performed on 250 μl PRP in a Siliconized cuvette with continuous stirring. The platelet count in the PRP was adjusted to 300,000 platelets/μl by dilution with PPP as needed. Aggregation was induced in PRP in response to collagen (10 μg/ml) (23). Prothrombin time and activated partial thromboplastin time were measured in a Hemochron-80 Dual Coagulation System (International Technidyne). Serum fibrinogen level and platelet count were measured by SRL.

Measurement of ACE and TF activities. In a separate set of animals, ACE activities of the intact common carotid artery were measured using a fluorometric assay (26). Tissue ACE activity was calculated as nanomoles of His-Leu generated per milligram of tissue weight per hour.

TF activities of the intact common carotid artery were also assessed with the use of the Tissue Factor Activity Kit (Product No. 846, ACTICHROME) according to the manufacturer's instructions. This assay measures intact TF as well as TF/factor VII and TF/factor VIIa complexes. Results are expressed in picomoles per milligram of tissue weight.

PCR analysis of TF mRNA. In a separate set of animals, total RNA from bilateral carotid arteries was reverse transcribed (Pharmacia Biotech), and the resultant cDNA was amplified by PCR with the following primers: sense primer 5′-GCAACCGAAACCCACCAACTAT-3′ and antisense primer 5′-AACCTAGTGTCTT-CCCGCGTG-3′. PCR was carried out with 20 cycles of denaturation at 93°C for 60 s, annealing at 60°C for 60 s, and polymerization at 72°C for 60 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified in the same way with the following primers: sense primer 5′-ACCACAGTCCATGCCATCAC-3′ and antisense primer 5′-TCCACCACCCTGTTGCT-GTA-3′. Each final PCR product sample was electrophoresed on a 0.8% agarose gel and visualized by ethidium bromide staining under ultraviolet (UV) light. UV signals were scanned by a densitometer. Relative gene expression was expressed as the ratio of TF mRNA to GAPDH mRNA.

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was performed using one-way ANOVA followed by Fisher's test for multiple comparison. A value of $P < 0.05$ was considered statistically significant.

Table 1. *Hemodynamic parameters*

	Systolic Blood Pressure, mmHg	Heart Rate, beats/min	Carotid Blood Flow, ml/min	Observed CFV/Animals Examined
Control group				
Baseline	117 ± 5	324 ± 29	5.9 ± 1.9	
75% Stenosis	123 ± 18	326 ± 39	5.1 ± 2.1	0/11
90% Stenosis	118 ± 12	323 ± 27	3.3 ± 2.6	0/9
Acute L group				
Baseline	152 ± 11*†	258 ± 37*†	2.9 ± 0.8*†	
Stenosis	152 ± 5*†	244 ± 37*†	2.3 ± 0.8*†	0/5
Chronic L group				
Baseline	152 ± 20*	315 ± 35	2.3 ± 0.9*	
Stenosis	150 ± 18*	317 ± 39		9/10
L+Hyd group				
Baseline	117 ± 15	348 ± 27	3.7 ± 0.6*	
Stenosis	118 ± 14	342 ± 40		4/5
L+Imi group				
Baseline	116 ± 5	325 ± 23	2.6 ± 0.6*	
Stenosis	116 ± 6	320 ± 16	1.9 ± 0.7*	0/6

Values are means ± SE. Rats were studied in the following groups: control, acute N^G-nitro-L-arginine methyl ester (L-NAME) treated (acute L), chronic L-NAME treated (chronic L), L-NAME and hydralazine treated (L+Hyd), and L-NAME and imidapril (L+Imi). CFV, cyclic flow variation (in ml/min). *$P < 0.01$ vs. the control group; †$P < 0.01$ vs. baseline values.

RESULTS

Compared with the control group, the acute L and chronic L groups showed a rise in systolic arterial pressure. In the L+Hyd and L+Imi groups, systolic arterial pressure did not change (Table 1). Basal CBF was less in the acute L, chronic L, L+Hyd, and L+Imi groups compared with the control group (Table 1).

Before introduction of stenosis, no CFVs were observed in any rats from all five groups. After 75% stenosis, CFVs were provoked 9 of 10 chronic L group animals and 4 of 5 L+Hyd group animals but none of control, acute L, or L+Imi group animals (Table 1). After more severe 90% stenosis, no control animals developed CFVs. Representative recordings of CFVs are shown in Fig. 1.

In another set of experiments, chronic L group rats displaying persistent CFVs for 30 min were randomly assigned to receive intravenous argatroban ($n = 6$), L-arginine ($n = 3$), or vehicle ($n = 6$). Administration of argatoroban, but not vehicle, significantly reduced the frequency and severity of CFVs (Figs. 2 and 3). Argatroban or vehicle did not affect systolic blood pressure or heart rate (data not shown). L-Arginine also reduced the frequency and severity of CFVs (data not shown).

The presence of obstructive thrombi were confirmed in all five chronic L group rats and 2 L+Hyd group rats that developed irreversible zero CBF after introduction of stenosis. Figure 4 shows microscopic pictures of carotid artery sections from the stenosis site from the

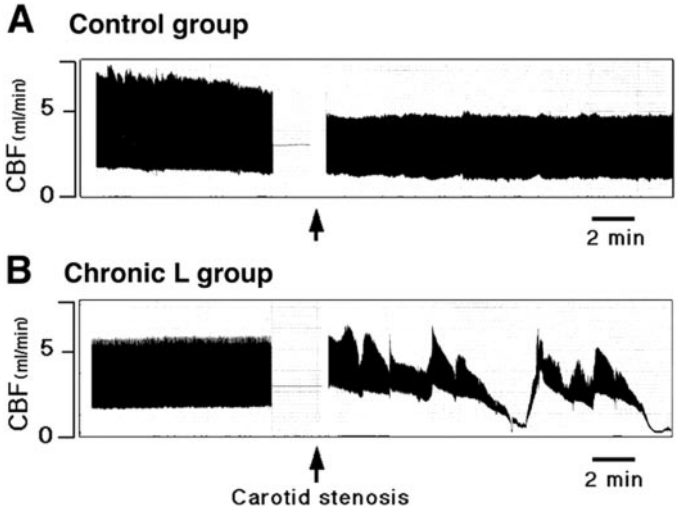

Fig. 1. Representative recordings of carotid blood flow (CBF) before and after production of 75% stenosis in a rat from the control group (*A*) and in a rat from the chronic N^G-nitro-L-arginine methyl ester (L-NAME)-treated (chronic L) group (*B*). Scale bar indicates 2 min.

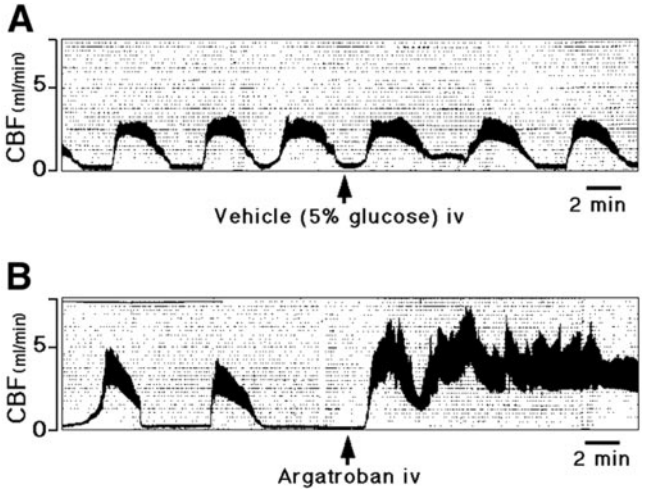

Fig. 2. Representative recordings showing the effects of vehicle (*A*) or argatoroban (*B*) on cyclic flow variations (CFVs) and the effects of vehicle (5% glucose) or argatroban on CFV frequency and CBF velocity. *A*: effects of vehicle on CFVs in a rat from the chronic L group; *B*: effects of argatroban on CFVs in a rat from the chronic L group. Scale bar indicates 2 min.

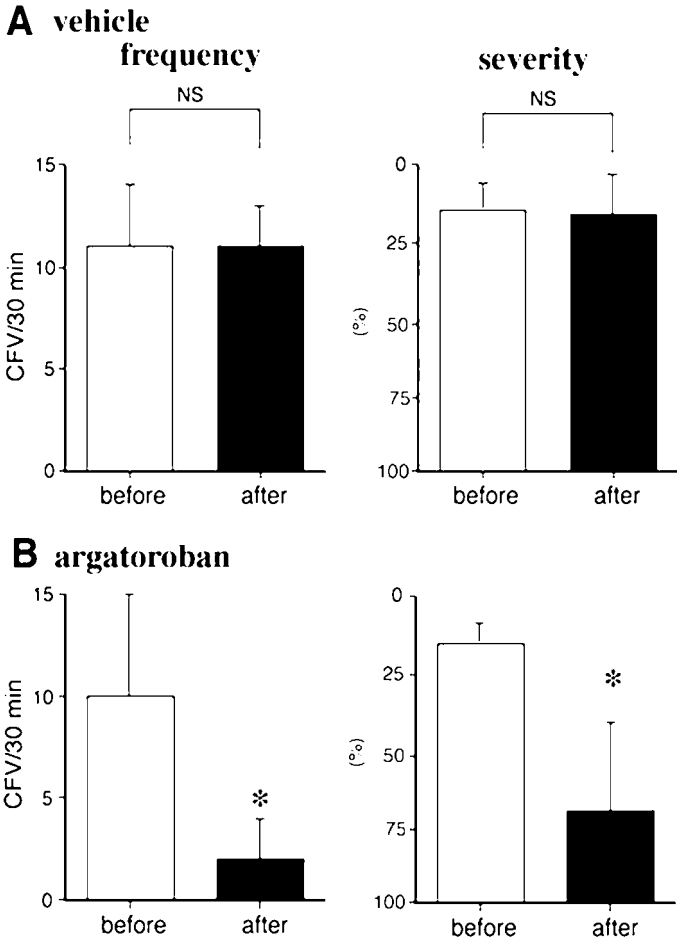

Fig. 3. Effect of the thrombin antagonist argatroban on the frequency and severity of CFVs in the chronic L group. *A*: effects of vehicle (5% glucose) on CFVs; *B*: effects of argatroban on CFVs. NS, not significant. *$P < 0.01$ vs. before treatment

chronic L groups. Platelet thrombus, which was attached to the damaged intimal surface, was seen at the site of stenosis. Careful histopathological analysis revealed the presence of injuries represented by disruption of endothelial cells and enfacement of the internal elastic membrane.

There were no significant differences in the medial thickening (the increase in the wall-to-lumen ratio) of carotid arteries among the control, chronic L, L+Hyd, and L+Imi groups (Table 2), suggesting that carotid arterial remodeling may not contribute to the development of CFVs.

Coagulation variables and platelet aggregation. Coagulation variables and platelet aggregation capacity were not significantly different among the control, chronic L, L+Hyd, and L+Imi groups ($n = 6$ each; Table 2). There were no significant difference between the control and chronic L groups concerning serum fibrinogen level and blood platelet count (Table 2).

Carotid ACE activity. As we reported (26), carotid tissue ACE activity was significantly increased in the chronic L and L+Hyd groups compared with the control group (Table 2). Treatment with imidapril prevented the L-NAME-induced increases in carotid ACE activity (Table 2). Compared with the control, serum ACE activity in the L or L+Hyd groups did not change but was suppressed in rats treated with imidapril (Table 2).

TF gene expression and activity. The carotid TF mRNA level was increased in the chronic L and L+Hyd groups (Fig. 5). The increased expression of TF mRNA was reduced in the L+Imi group. Tissue TF activity was increased in the chronic L group (Table 2). The increased TF activity was prevented by treatment with imidapril but not with hydralazine.

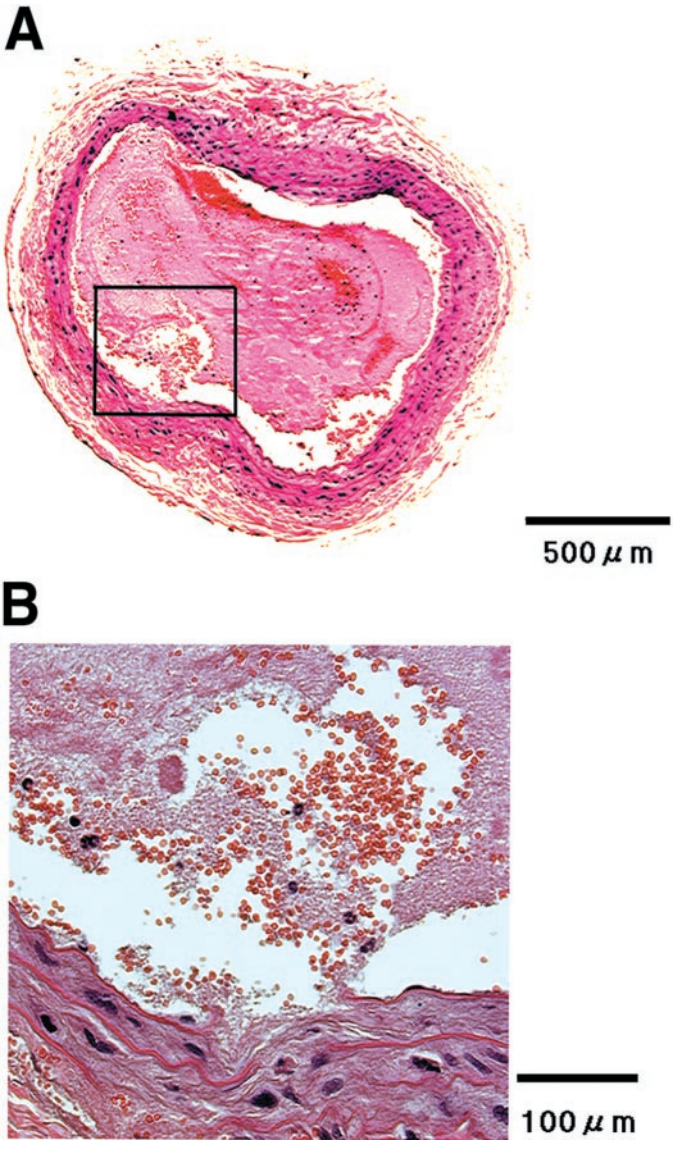

Fig. 4. Micrographs of carotid artery cross sections at the site of stenosis from the chronic L group stained with hematoxylin-eosin. *A*: platelet thrombus is present in the carotid artery from a rat of the chronic L group that developed irreversible zero flow after introduction of stenosis. Scale bar indicates 500 μm. *B*: expanded view of area indicated in *A*. Injuries with ablation of endothelial cells and disruption of internal elastic membrane are seen. Scale bar indicates 100 μm.

Table 2. *Platelet aggregation, coagulation variables, ACE activity, and vascular remodeling*

		Groups			
	n	Control	Chronic L	L+Hyd	L+Imi
Platelet count, $\times 10^4$/mm^3	6	69 ± 7	62 ± 3	64 ± 2	67 ± 3
Platelet aggregation, %	6	60 ± 4	52 ± 5	63 ± 2	62 ± 1
PT, s	6	12 ± 0.1	12 ± 0.2	12 ± 0.3	12 ± 0.2
APTT, s	6	25 ± 3	30 ± 2	31 ± 2	31 ± 2
Fibrinogen, mg/dl	6	173 ± 23	169 ± 14	198 ± 9	213 ± 10
Serum ACE activity, nmol·ml^{-1}·h^{-1}	6	0.8 ± 0.2	0.8 ± 0.3	0.8 ± 0.4	0.1 ± 0.1*†
Tissue ACE activity, nmol·mg^{-1}·h^{-1}	6	1.0 ± 0.2	2.5 ± 0.3*	2.4 ± 0.3*	0.3 ± 0.1*†
Tissue tissue factor activity, pmol/g	6	16.1 ± 0.7	22.4 ± 2.0*	22.0 ± 1.0*	16.9 ± 0.9
Wall-to-lumen ratio	5	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01

Values are means ± SE; n = number of rats. PT, prothrombin time; APPT, activated partial PT; ACE, angiotensin-converting enzyme. *$P < 0.01$ vs. the control group; †$P < 0.01$ vs. the chronic L group.

DISCUSSION

We report here that long-term inhibition of NO synthesis by treatment with L-NAME increases thrombogenicity of the rat carotid artery. The increased thrombogenecity was not associated with systemic coagulation parameters but with increased gene expression and activity of TF and was reduced exquisitely by argatroban. We further show that ACE inhibition prevented the development of thrombogenecity and attenuated increased TF expression. These observations show the importance of angiotensin II-mediated induction of TF and the resultant thrombin generation in the pathogenesis of thrombus formation after stenosis-related arterial damage in this model.

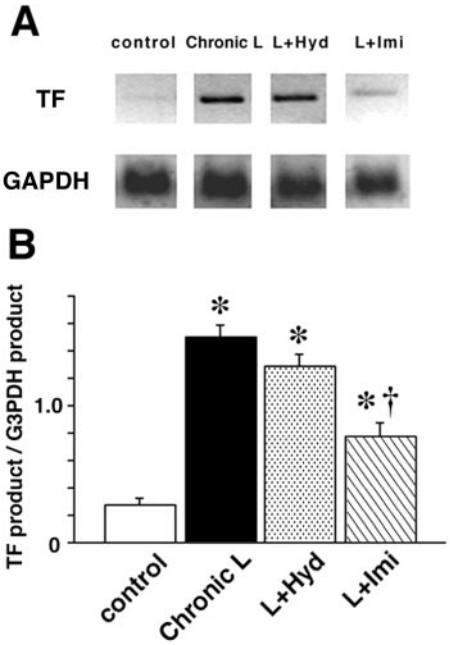

Fig. 5. Tissue factor (TF) mRNA levels in carotid arteries. *A*: expression of carotid TF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in rats from the control group, the chronic L group, L-NAME and hydralazine-treated (L+Hyd) group, and the L-NAME and imidapril-treated (L+Imi) group by RT-PCR. *B*: densitometric analysis of data in *A*. Expression of TF mRNA in each sample was normalized relative to GAPDH mRNA expression in that sample. *$P < 0.01$ vs. the control group; †$P < 0.01$ vs. the chronic L group.

CFVs have been used by many investigators as a reliable marker of recurrent platelet-rich thrombus formation (9, 20, 33). The pathogenesis of CFVs has been studied extensively in the model of Folts et al. (9), in which CFVs occurs immediately after stenosis with severe endothelial damages in the normal artery. CFVs are believed to reflect growth and dislodgment of arterial thrombi and have been observed in patients during the course of acute coronary syndrome (1). The occurrence of occlusive thrombus was apparent in animals displaying irreversible zero flow in this study as well. Histopathological analysis suggests that disruption of the endothelial layer and subintimal elastic membrane and subsequent enfacement of subendothelial components to platelets might trigger CFVs (Fig. 4). In the present study, CFVs occurred in rats receiving L-NAME for 4 wk but not in control rats, or rats receiving acute L-NAME administration. The absence of CFVs in controls may be due to less-extensive endothelial damage in our present model than in the model of Folts et al. (9). Our present data indicate the essential role of TF-mediated generation of thrombin from the arterial wall in the cause of thrombosis in our present model. Overall, stenosis-related vascular damage triggered interaction of the locally produced thrombin in subintimal area with circulating platelets, which in turn caused thrombus in the present study. In the model of Folts et al. (9) with more-extensive endothelial damage to the intact artery, the platelet dependency of CFVs has been emphasized by the effectiveness of antiplatelet drugs (9). In contrast, TF-mediated generation of thrombin appears to play an important role in thrombogenesis in the present model.

We have previously shown that an activation of ACE with resultant formation of angiotensin II is vital in the pathogenesis of early vascular inflammation and late cardiovascular remodeling after long-term inhibition of NO synthesis in rats (14–16, 26, 30). Angiotensin II is known to activate the coagulation cascade in the blood vessel wall. Because TF is recognized as an essential initiator of thrombin generation and coagulation in vascular wall (24) and angiotensin II has been shown to increase TF production (21, 27), we tested the hypothesis that ACE inhibition can reduce increased thrombogenecity through a decrease in TF gene ex-

pression and activity in animals with chronic administration of L-NAME. TF-driven thrombin generation was assumed to play a pivotal role in the thrombogenicity of the atherosclerotic plaque (i.e., acute coronary syndrome) and possibly in restenosis after percutaneous revascularization (29). In the present study, ACE inhibition attenuated the increases in TF gene expression and activity as well as the occurrence of CFVs induced by chronic L-NAME administration. Hydralazine normalized L-NAME-induced hypertension but did not affect the thrombogenecity. Although basal CBF was less in acute L, chronic L, and L+Hyd groups than in the control group, CFVs were observed only in the chronic L group and L+Hyd groups. Furthermore, severe 90% stenosis did not cause CFVs in the control group. Therefore, it is unlikely that arterial hypertension or a decrease in CBF induced by L-NAME contributed largely to the pathogenesis of CFVs in rats with long-term inhibition of NO synthesis. These results suggest that TF-mediated generation of thrombin through increased angiotensin II activity may be vital in mediating thrombosis after stenosis-related vascular damages in rats with long-term blockade of NO synthesis.

It has been shown by many investigators that TF expression and/or activity in the diseased arterial wall may be mainly from monocytes/macrophages that infiltrated into the diseased arterial wall (2, 29, 32). Accumulation of ACE in areas of monocytes/macrophages in atherosclerotic plaques and angiotensin II-induced TF expression have been reported (5, 19). We previously reported increases in oxidative stress, NF-κB activity, infiltration of monocytes, and MCP-1 production in arterial tissues of rats received chronic L-NAME administration (15). Treatment with a NF-κB decoy or blockade of MCP-1 attenuated monocyte infiltration into the vascular wall and the subsequent arteriosclerosis (8, 15, 16). Treatment with ACE inhibitor, angiotensin II type 1 receptor antagonist, or antioxidants prevented all of these changes (30). Thus our present data may provide a new link between tissue ACE activity and thrombogenecity in this model.

In conclusion, our present data support the hypothesis that long-term inhibition of endothelial NO synthesis may increase arterial thrombogenecity at least in part through angiotensin II-induced induction of TF and the resultant thrombin generation. These data may provide new insights as to how endothelial NO contributes to antithrombogenic properties of the vascular endothelium in vivo.

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