Very-Low-Dose Combination of the Angiotensin-Converting Enzyme Inhibitor Perindopril and the Diuretic Indapamide Induces an Early and Sustained Increase in Neovascularization in Rat Ischemic Legs

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ABSTRACT
After acute ischemia of tissues, neovascularization must be sufficient and fast enough to preserve tissue integrity and organ function, and may thus be considered as a therapeutic strategy. This study examined the possible role of the very-low-dose combination of perindopril (angiotensin-converting enzyme inhibitor) and indapamide (diuretic), used first-line in the treatment of essential hypertension, on ischemia-induced angiogenesis. Ischemia was produced by artery femoral occlusion in rats treated or not with the very-low-dose combination (perindopril 0.76 mg/kg/day + indapamide 0.24 mg/kg/day) or each component given alone at the same dosage for 3 and 28 days. At day 3, angiographic vessel density and laser Doppler perfusion data showed significant improvement in ischemic/non-ischemic leg ratio by, respectively, 1.9-fold and 1.5-fold in rats treated with the very-low-dose combination when compared with controls (p < 0.05). This was associated with an increase in vascular endothelial growth factor (VEGF; 2.2-fold) and endothelial nitric-oxide synthase (1.6-fold) protein content in the ischemic hindlimb, assessed by Western blot. At day 28, the very-low-dose combination (3- and 1.6-fold) and perindopril alone (1.8- and 1.4-fold) and indapamide alone (2.0- and 1.4-fold) increased the angiographic score and blood flow perfusion, respectively, in reference to controls (p < 0.05). Furthermore, addition of VEGF-neutralizing antibody (2.5 μg/kg twice a week) or NOS inhibitor (N^G-nitro-L-arginine methyl ester, 10 mg/kg/day) prevented the pro-angiogenic effect induced by the perindopril/indapamide combination. The very-low-dose combination of perindopril and indapamide induces an early and sustained effect on the revascularization process observed in ischemic tissue and may provide a favorable therapeutic neovascularization after ischemia.

The ability of organisms to spontaneously develop collateral vessels represents an important response to occlusive diseases and determines the severity of residual tissue ischemia. In the setting of ischemia, the formation of new capillary blood vessels is under the control of both hypoxia and inflammation (Carmeliet, 2000). The main mechanism of hypoxia-induced angiogenesis involves the rise in hypoxia-inducible factor-1 protein leading to the up-regulation of the gene encoding vascular endothelial growth factor (VEGF) (Shweiki et al., 1992; Carmeliet, 2000). VEGF has been shown to trigger the cascade of angiogenesis through, in part, activation of endothelial nitric-oxide synthase (eNOS) and NO-related pathways (Murohara et al., 1998b). Neovascularization appears to be also controlled by the inflammatory process that occurs during vessel growth in ischemic tissues (Sunderkotter et al., 1991; Arras et al., 1997; Silvestre et al., 2000). The presence of inflammatory cells is associated with local secretion of several factors, including cytokines, growth factors, and matrix metalloproteinases resulting in neangiogenesis (Sunderkotter et al., 1991; Arras et al., 1997).

In the last decade, therapeutic strategies designed to augment native collateral vessel growth have been developed to achieve perfusion of ischemic tissue. Such strategies were applied in animal models of limb or myocardial ischemia and tested even at the clinical level in treatment of patients with peripheral vascular obstruction or coronary artery diseases (Baumgartner et al., 1998; Losordo et al., 1998; Kalka et al., 2000; Laham et al., 2000; Lazarous et al., 2000). Nevertheless, clinical strategies using growth factors, including VEGF and fibroblast growth factor, or endothelial progenitor cells may harbor potential hazards and are currently under clin-

ABBREVIATIONS: VEGF, vascular endothelial growth factor; eNOS, endothelial nitric-oxide synthase; NO, nitric oxide; ACE, angiotensin-converting enzyme.
iological investigation for therapeutic angiogenesis (Epstein et al., 2001). In addition, the ways that potentially therapeutic agents are delivered to patients are either crude (intramuscular administration, adeno-virally mediated transfections of cells) or require sophisticated manipulation. Finally, such strategies could also lead to deleterious effects, especially in patients with coexistent diseases, such as atherosclerosis.

Therefore, one potential alternative strategy may be the use of drugs with pro-angiogenic activity, available in an oral formulation, which are currently administered to patients for treatment of different pathologies. Perindopril and indapamide are both well established agents that are effectively used as first-line treatment for hypertension (Matheson et al., 2001). Perindopril is a long-acting angiotensin-converting enzyme (ACE) inhibitor, and indapamide is an indoline derivative of chlorsulfonamide that has both diuretic and anti-hypertensive properties (Matheson et al., 2001). Both agents display alternative biological effects that may improve overall therapeutic efficacy and may open the way for their use in alternative diseases. The very-low-dose perindopril/indapamide combination raised capillary density in hypertensive rats compared with untreated hypertensive animals (Levy et al., 2001). Similarly, this very-low-dose combination increased coronary capillary density in stroke-prone hypertensive rats (Rakusan et al., 2000). ACE inhibition has also been shown to promote angiogenesis in ischemic hindlimb of normotensive animals (Fabre et al., 1999; Silvestre et al., 2001).

We therefore hypothesized that the very-low-dose combination of the ACE inhibitor perindopril and the nonselective diuretic indapamide may improve ischemia-induced neovascularization. We analyzed the revascularization process associated with ischemia (Iglarz et al., 2001). We also assessed the effect of the nonspecific vasodilator hydralazine hydrochloride (200 mg/l in drinking water).

Systolic arterial blood pressure was assessed by the tail-cuff method (BP recorder 8006; W+W Electronic, Apelex, France).

**Quantification of Angiogenesis**

**Microangiography.** Vessel density was evaluated by high-definition microangiography at the end of the treatment period, as previously described (Silvestre et al., 2000). Briefly, animals were anesthetized (pentobarbital, 50 mg/kg i.p.) and a contrast medium (barium sulfate, 1 g/ml) was injected through a catheter introduced into the abdominal aorta. Images acquired by a digital X-ray transducer were assembled to obtain a complete view of the hindlimbs (Fig. 1). The angiographic score was expressed as a percentage of pixels per image occupied by vessels in the quantification area.

**Capillary Density.** Microangiographic analysis was completed by assessment of capillary density, as previously described (Silvestre et al., 2000). Ischemic and nonischemic muscles were dissected and progressively frozen in isopentane solution cooled in liquid nitrogen. Sections (7 μm) were incubated with rabbit polyclonal antibody directed against total fibronectin (dilution 1:50; TEBU, Yvelines, France) to identify capillaries (Fig. 1). Capillary density was then assessed with antibody directed against total fibronectin (Fig. 1).

**Materials and Methods**

**Animal Model: Rat Ischemic Hindlimb Model**

This study was conducted in accordance with both institutional guidelines and those formulated by the European Community for experimental animal use (L358-86/609EEC). Twelve-week-old male normotensive Wistar rats (Iffa-Credo, L’Arbresle, France) were used for this study. The right femoral artery was occluded (3–0 silk suture) under pentobarbital anesthesia (50 mg/kg i.p.). The ligature was performed on the femoral artery 0.5 cm proximal to the bifurcation of the saphenous and popliteal arteries, as previously described (Silvestre et al., 2000).

**Set of Experiments 1.** Rats were then treated for 3 (n = 5) or 28 days (n = 6) with the very-low-dose combination of perindopril and indapamide (1 mg/kg/day, i.e., 0.76 mg/kg/day and 0.24 mg/kg/day, respectively) or perindopril (0.76 mg/kg/day) in the drinking water; Servier, France, Courbevoie, France, or with indapamide (0.24 mg/kg/day in powder chow; Servier, France). Untreated rats served as control groups.

**Set of Experiments 2.** We next analyzed the involvement of the VEGF/NO synthesis inhibitor, A(1)-arginine, in the effect of the perindopril/indapamide combination. Rats were then treated for 21 days (n = 5) with a combination of perindopril/indapamide + VEGF-neutralizing antibody (2.5 μg i.p. twice a week; R & D Systems Europe, Oxford, UK) or with a combination of perindopril/indapamide + a nonselective NO synthesis inhibitor, A(1)-nitro-L-arginine methyl ester (10 mg/kg/day in drinking water; Sigma, Saint Quentin Fallavier, France).

Such doses have already been shown to block the revascularization process associated with ischemia (Iglarz et al., 2001). We also assessed the effect of the nonselective vasodilator hydralazine hydrochloride (200 mg/l in drinking water).

Fig. 1. A, representative microangiography of the right ischemic and left nonischemic hindlimbs, 28 days after femoral artery occlusion in rats. B, representative photomicrographs of ischemic muscle sections hybridized with antibody directed against total fibronectin, 28 days after ischemia. Capillaries appear in white and myocytes in black. Cont, controls; Per+Ind, perindopril and indapamide-treated rats.

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calculated in a randomly chosen field of a definite area, using Opti-
lab/Pro software.

Laser Doppler Perfusion Imaging. To provide functional evidence
for ischemia-induced changes in vascularization, laser Doppler perfusion
imaging experiments were performed in rats, as previously described (Sil-
vestre et al., 2001). Briefly, excess hairs were removed by depilatory cream
from the limb before imaging, and rats were placed on a heating plate at
37°C to minimize temperature variation. Nevertheless, to account for vari-
ables, including ambient light and temperature, calculated perfusion was
expressed as a ratio of ischemic to nonischemic leg.

**Determination of VEGF and eNOS Protein Expression**

VEGF and eNOS protein expression was determined by Western
blot in ischemic and nonischemic legs, as previously described (Sil-
vestre et al., 2001).

**Statistical Analysis**

Results are expressed as mean ± S.E.M. One-way analysis of
variance was used to compare each parameter. Post hoc Bonferonni’s
\(t\) test comparisons were then performed to identify which group

differences account for the significant overall analysis of variance. A
value of \(p < 0.05\) was considered significant.

**Results**

**Hemodynamic Parameters**

At day 3 of treatment, administration of indapamide alone,
perindopril alone, or the very-low-dose combination of perin-
dopril/indapamide did not affect systolic blood pressure when
compared with untreated control (141 ± 4 mm Hg, 138 ± 7
mm Hg, and 139 ± 6 mm Hg versus 138 ± 5 mm Hg, \(p =
0.83\)). Similarly, at day 28, no significant changes in systolic
blood pressure were observed in either group (139 ± 3 mm
Hg, 138 ± 7 mm Hg, and 140 ± 3 mm Hg versus 141 ± 4 mm
Hg, for indapamide-, perindopril-, and perindopril + indap-
amide-treated rats versus control, respectively; \(p = 0.71\)).

**Very-Low-Dose Combination Increased Ischemia-Induced
Angiogenesis in Rat Hindlimbs**

**Microangiography.** At day 3 of treatment, administration of indapamide alone
or perindopril alone did not affect the angiographic score. In contrast, the very-low-dose combina-
tion of perindopril/indapamide raised by 1.9-fold the ischemic/nonischemic leg ratio when compared with that of controls (\(p < 0.05\)). At day 28, this very-low-dose combination (3-fold), indapamide alone (2.0-fold), and perindopril alone (1.8-fold) increased the angiographic score in reference to control animals (\(p < 0.05\)) (Figs. 1 and 2). No significant changes were observed in the ischemic hindlimb whatever the time of treatment in either group (data not shown). Interestingly, treatment with the nonspecific vasodilator hydralazine hydrochloride did not affect vessel density when compared with untreated controls (\(p > 0.05\); see Fig. 5).

**Capillary Density.** Microangiographic data were confirmed
by capillary density analysis. At day 3, the very-low-dose com-
bination of perindopril/indapamide increased by 2.2-fold the ischemic/nonischemic capillary number ratio when compared with that of controls (\(p < 0.05\)). Conversely, treatment with indapamide alone or perindopril alone did not affect capillary density. At day 28, the combination (1.9-fold), indapamide alone (1.6-fold), and perindopril alone (1.6-fold) enhanced the ischemic/nonischemic capillary number ratio when compared with that of controls (\(p < 0.05\)) (Figs. 1 and 2). No significant changes were observed in the nonischemic hindlimb whatever the time

**Blood Flow Perfusion.** Microangiographic and capillary
density measurements were associated with changes in blood

\[ \text{Fig. 2. A, ischemic/nonischemic angiographic score ratio. B, ischemic/}
\text{nonischemic capillary density ratio. C, ischemic/nonischemic cutaneous}
\text{blood flow ratio. Values are mean ± S.E.M. *}, \text{p < 0.05 versus control rats.}
\text{Cont, controls; Ind, indapamide-treated rats; Per, perindopril-treated}
\text{animals; and Per + Ind, perindopril and indapamide-treated rats.} \]
perfusion. At day 3, the ischemic/nonischemic leg ratio was increased by 1.5-fold in perindopril/indapamide-treated rats compared with untreated animals ($p < 0.05$). Such a ratio was unaffected by treatment with indapamide alone or perindopril alone (Fig. 2). At day 28, administration of the very-low-dose combination of perindopril/indapamide, indapamide alone, and perindopril alone enhanced by 1.6-, 1.4-, and 1.4-fold, respectively, the ischemic/nonischemic leg blood perfusion ratio ($p < 0.05$ versus controls). In addition, hydralazine hydrochloride did not affect blood perfusion when compared with untreated animals ($p > 0.05$; see Fig. 5).

**Molecular Mechanisms of Very-Low-Dose Combination-Induced Angiogenesis**

**VEGF.** At day 3, in the nonischemic leg, VEGF protein level was unaffected in either group. In contrast, in the ischemic hindlimb, the very-low-dose combination of perindopril/indapamide, indapamide alone, and perindopril alone up-regulated VEGF protein content by 126%, 82%, and 46%, respectively, over that of untreated controls ($p < 0.05$). At day 28, in the nonischemic leg, VEGF protein level was unchanged in either group. In the ischemic leg, only the very-low-dose combination of perindopril/indapamide enhanced by 69% the VEGF protein level ($p < 0.05$) (Fig. 3). Furthermore, treatment with VEGF-neutralizing antibody prevented the pro-angiogenic effect induced by the very-low-dose combination treatment, confirming the requirement of VEGF in the angiogenic process (see Fig. 5).

**eNOS.** At day 3, in the nonischemic leg, eNOS protein level was unaffected in either group. In the ischemic hindlimb, the very-low-dose combination of perindopril/indapamide increased by 61% eNOS protein content in reference to untreated controls ($p < 0.05$). At day 28, in the nonischemic leg, eNOS protein level was unchanged in either group. Administration of indapamide alone and perindopril alone enhanced by 50% and 49% eNOS levels when compared with untreated controls (Fig. 4). The very-low-dose combination of perindopril/indapamide also raised by 83% eNOS protein content over that of untreated rats ($p < 0.05$). In addition, the increase in vessel density and blood flow perfusion observed in perindopril/indapamide-treated rats was blocked by NOS inhibitor treatment, emphasizing the key role of eNOS in the angiogenic reaction (Fig. 5).

**Discussion**

In this study, we showed that a very-low-dose combination of perindopril/indapamide induces an early and sustained neovascularization process in ischemic leg through an activation of VEGF/eNOS signaling.

Therapeutic angiogenic strategies using growth factors or endothelial progenitor cells may cause harmful side effects (Epstein et al., 2001). Among these, the potent vascular permeability activity of VEGF is likely to have several undesirable consequences. Transient edema is observed in 34% of patients administered VEGF (Baumgartner et al., 2000). Another unfavorable consequence of VEGF therapy is that the newly formed vessels can be functionally abnormal. Indeed, delivery of VEGF in ischemic tissues rescues blood perfusion but induces formation of immature vascular structure (Isner et al., 1996; Murohara et al., 1998a). Angiographic studies clearly show that the newly formed vasculature is not well organized as in normal tissues, resembling the characteristics of leaky hemangiomata (Isner et al., 1996; Murohara et al., 1998a). Growth factors are also present in atherosclerotic plaques, and under various experimental circumstances, their administration increases neointimal smooth muscle cell proliferation and neointimal mass (Nabel et al., 1993; Inoue et al., 1998). Similarly, administration of bone marrow-derived progenitor endothelial cells by secreting potent angiogenic ligands and cytokines could also lead to deleterious effects (Davidoff et al., 2001).

Drug therapy may provide an additional strategy to angiogenic growth factor therapies (protein and gene), which may be advantageous because these drugs are orally administered and known to be safe in humans. In this view, HMG-CoA (3-hydroxy-3-methylglutaryl CoA) reductase inhibitors...
Statins have been developed as lipid-lowering drugs and are well established to reduce morbidity and mortality from coronary artery disease. Besides lipid-lowering, statins are capable of stimulating the growth of new blood vessels in rabbit ischemic limbs (Kureishi et al., 2000). Statins activate Akt, increase phosphorylation of the endogenous Akt substrate eNOS, inhibit apoptosis, and accelerate vascular structure formation in an Akt-dependent manner (Kureishi et al., 2000). Statins also augment the number and the differentiation of endothelial progenitor cells, which may contribute to statin-induced increase in the revascularization process after tissue ischemia (Dimmeler et al., 2001). In our present study, we provide evidence that two other drugs, perindopril and indapamide, up-regulate the revascularization process in the ischemic tissue. Moreover, the very-low-dose combination of perindopril and indapamide promotes an early and sustained pro-angiogenic effect when compared with administration of perindopril alone or indapamide alone, suggesting that both agents may have an additive or synergic effect. Interestingly, no changes in angiographic score were observed in the nonischemic legs. It is likely that ischemic tissue responds more sensitively to biological effects of angiogenic factors. This concept is supported by a study in which exposure to high local levels of fibroblast growth factor-1 induced an angiogenic response in ischemic canine myocardium but not in nonischemic tissue (Banai et al., 1991). In an attempt to investigate the potential mechanisms involved in perindopril/indapamide-related neovascularization, we examined the effect of each agent on the VEGF/eNOS pathway. The increase in the revascularization process observed in perindopril- and/or indapamide-treated rats was associated with local up-regulation of VEGF and eNOS expression. Furthermore, addition of VEGF-neutralizing antibody or NOS inhibitor prevented the pro-angiogenic effect induced by perindopril/indapamide treatment.

Hence, VEGF/eNOS expression and signaling promote, at least in part, angiogenesis in this setting, as previously reported in models of ischemic injury (Murohara et al., 1998b; Matsunaga et al., 2000). Previous studies have already reported a correlation between perindopril/indapamide treatment and NO-related pathways. The very-low-dose combination of perindopril and indapamide alone raised NOS activity in vessels of hypertensive rats (Hayakawa et al., 1997). Both agents may also directly or indirectly modulate several cellular pathways that play an important role in the regulation of new vessel growth. ACE inhibition has been shown to activate eNOS expression by stimulating bradykinin B2 receptor pathway (Silvestre et al., 2001). Similarly, indapamide potentiates bradykinin-related actions on femoral artery (Junquero et al., 1991). Interestingly, bradykinin is a potent activator of ischemia-induced angiogenesis (Emanuelli et al., 2001). Indapamide also raises prostaglandin generation, which has been reported to affect VEGF gene expression and, subsequently, the angiogenic process (Junquero et al., 1991; Tsuji et al., 1998). Nevertheless, the driving mechanisms that lie behind the increase in VEGF and eNOS expression induced by each component of this very-low-dose combination remain to be clarified.

Therefore, this very-low-dose combination has powerful effect on revascularization associated with ischemia, inducing an early and sustained pro-angiogenic effect through a local activation of VEGF/eNOS signaling. This very-low-dose combination of perindopril and indapamide may provide a promising and well tolerated treatment option in the management of arterial hypertension associated with peripheral vascular obstruction or coronary artery diseases.

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