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PARADOXICAL ROLE OF ANGIOTENSIN II TYPE 2 RECEPTORS IN RESISTANCE ARTERIES OF OLD RATS.

#Frederic Pinaud, M.D., Arnaud Bocquet, Ph.D., Odile Dumont, B.Sc., Kevin Retailleau, B.Sc, #Christophe Baufreton, M.D., Ph.D., Ramaroson Andriantsitohaina, Pharm.D., Ph.D., Laurent Loufrani, Ph.D., Daniel Henrion, Pharm.D., Ph.D

short title: VASCULAR TONE IN AGING

CNRS UMR 6214; INSERM UMR 771; Université d'Angers;

CHU d'Angers, Dept of Cardiac Surgery and UPRES-EA 3860, Angers, France

Address for Correspondence:

Dr. Daniel Henrion, Pharm.D., Ph.D.

Dept of Neurovascular Integrated Biology - UMR CNRS 6214-INSERM 771

Faculté de Médecine, 49045 Angers, FRANCE

tel: 332 41 73 58 45 fax: 332 41 73 58 95

E-mail: daniel.henrion@univ-angers.fr

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Abstract

The role of angiotensin II type 2 receptors (AT2R) remains a matter of controversy. Its vasodilatory and antitrophic properties are well accepted. Nevertheless, in hypertensive rats AT2R stimulation induces a vasoconstriction counteracting flow-mediated dilation (FMD). This contraction is reversed by hydralazine. As FMD is also decreased in aging, another risk factor for cardiovascular diseases, we hypothesized that AT2R function might be altered in old rats resistance arteries.

Mesenteric resistance arteries (250 μm diameter) were isolated from old (24 months) and control (4 months) rats receiving hydralazine (16 mg/kg/day, 2 weeks) or water. FMD, NO-mediated dilation and eNOS expression were lower in old than in control rats. AT2R blockade improved FMD in old rats, suggesting that AT2R stimulation produced vasoconstriction. AT2R expression was higher in old rats and mainly located in the smooth muscle layer. In old rats AT2R stimulation induced endothelium-independent contraction, which was suppressed by the antioxydant Tempol. Reactive oxygen species (ROS) level was higher in old rats arteries than in controls. Hydralazine improved FMD and NO-dependent dilation in old rats without change in AT2R expression and location. In old rats treated with hydralazine ROS level was reduced in endothelial and smooth muscle cells and AT2R-dependent contraction was abolished.

Thus, AT2R stimulation induced vasoconstriction through activation of ROS production, contributing to decrease FMD in old rats resistance arteries. Hydralazine suppressed AT2R-dependent ROS production and AT2R-dependent contraction, improving FMD. Importantly, endothelial alterations in aging were reversible. These findings are important to consider in the choice of vasoactive drugs in aging.

Key words: aging, endothelium, microcirculation, angiotensin II receptors, nitric oxide, oxidative stress, vasodilator agents

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INTRODUCTION

Resistance arteries play a key role in vascular homeostasis. They possess a basal vasoconstrictor tone [1, 2] counteracted in part by flow (shear stress)-mediated dilation (FMD). Their tone is modified in hypertension, ischemic diseases, myocardial infarction or diabetes; situations being more frequent in aging [3-5]. In aging, large arteries develop calcification and become less compliant [6]. By contrast, resistance arteries do not develop calcification or stiffening in old subjects nevertheless, the microvascular network becomes less efficient [1]. Indeed, aging reduces the ability to increase blood flow to skeletal muscle, as shown in rats [7] and humans [8], due to endothelial dysfunction [3]. Endothelium-dependent contraction is increased in aging in parallel with a decreased endothelial capacity to produce vasodilator agents [9]. In humans, FMD, assessed by forearm blood flow measurement after reactive hyperemia, is lowered [10-12].

We have previously shown that angiotensin II type 2 receptor (AT2R) is involved in FMD in normotensive rats resistance arteries [13] whereas in hypertensive animals FMD is counteracted by angiotensin II type 1 receptor (AT1R) and AT2R-induced vasoconstriction [2, 14]. As hypertension is often presented as a premature vascular aging we tested the hypothesis that in aging AT2R receptor function might also be altered in resistance arteries. Thus, we determined the involvement of AT2R dependent-tone in FMD and determined the pathway stimulated after stimulation of AT2R in resistance arteries isolated from old rats. In addition, as we have previously shown that a chronic treatment with hydralazine reverses AT2R-dependent contraction into dilation in hypertensive rats [2], we also tested the hypothesis that hydralazine might improve AT2R-dependent tone and/or FMD in old rats. We used a dose of hydralazine previously shown to improve mesenteric blood flow without changing systemic blood pressure [15].

MATERIALS AND METHODS

Animals

Twelve old (24 months) and 12 young (4 months) male Wistar rats (Iffa-Credo, L'Arbresle, France) were treated for 2 weeks with hydralazine (16 mg/kg per day in drinking water) [15] or tap water (control). They were then anesthetized with isoflurane. Arterial blood pressure was measured in the femoral artery. Their mesentery was then removed in order to isolate mesenteric resistance arteries (MRA).

The procedure followed in the care and euthanasia of the study animals was in accordance with the European Community Standards on the Care and Use of Laboratory Animals (Ministère de

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l'Agriculture, France, authorization # 6422). From each rat several segments of mesenteric arteries were isolated for the following experiments.

Pressure and flow-dependent tone in mesenteric resistance arteries

From each rat a segment of third order MRA was cannulated and mounted in a video monitored perfusion system and myogenic tone measured. Flow-mediated dilation was then determined before and after nitric oxide (NO) synthesis or AT2R blockade. At the end of the experiment passive diameter of the vessel, i.e., in the absence of smooth muscle tone was measured. (please see online supplement at http://hyper.ahajournals.org)

Vascular response to exogenous angiotensin II in isolated mesenteric arteries.

Six segments of MRA were used per rat (12 rats per group) in order to test the effect of endothelium removal, NO synthesis blockade with NG-nitro-L-arginine methyl ester (L-NAME 100 µmol/L), cycloxygenase blockade with indomethacin (10 µmol/L), bradykinin B2 receptor blockade with HOE 140 (10 µmol/L), AT2R blockade with PD 123319 (10 µmol/L) or reactive oxygen species (ROS) removal with the catalase mimetic Tempol (100 µmol/L) on AT2-dependent dilation or contraction. (please see online supplement at http://hyper.ahajournals.org)

Western Blot Analysis of AT2 Receptors and endothelial NO Synthase (eNOS) expression and Immuno-histological analysis of AT2 Receptors (please see online supplement at http://hyper.ahajournals.org)

Detection of ROS using confocal microscopy in resistance arteries

ROS detection was performed on transverse cross sections 7 µm thick incubated with dihydroethydine (DHE), as previously described [16]. Positive staining using confocal microscopy and image analysis was performed as described above.

Statistical analysis

Results were expressed as means ± s.e.m. Significance of the differences between groups was determined by analysis of variance: 2 factors ANOVA analysis on the whole curve or one way ANOVA analysis followed by a Bonferroni test. P values less than 0.05 were considered to be significant.

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RESULTS

AT2R blockade with PD123319 (10 µmol/L) significantly reduced FMD in young rats (figure 1A) and significantly increased FMD in old rats (figure 1B). In old rats the effect of PD123319 on FMD was more pronounced in the range of low flow rates (until 21 µl/min, figure 1B). After treatment with hydralazine in young rats, PD123319 also reduced FMD (figure 1C). This effect of PD 123319 was not significant using a 2 factor ANOVA analysis on the whole curve but a significant effect was found for the flow range 15-30 µl/min when using a one way ANOVA analysis followed by a Bonferroni test.
In old rats treated with hydralazine, PD123319 had no significant effect on FMD (figure 1D). Western blot analysis performed in MRA showed that AT2R expression was significantly higher in old than in young rats. Hydralazine had no significant effect on AT2R expression level in old rats but significantly increased AT2R expression in young rats (figure 1E).

In MRA, AT2R was visualized using confocal microscopy and fluorescent angiotensin II in the presence of candesartan. AT2R were present in both the endothelium and the tunica media in young and old rats. Nevertheless, in old rats, AT2R density in the endothelium was 3 times lower than in young rats (figure 2). Hydralazine had no significant effect on AT2R location (figure 2). Negative control (in the presence of PD 123319 or in the absence of fluorescent angiotensin II) showed the absence of labeling.

Acute stimulation of AT2R with angiotensin II in the presence of candesartan induced dilation in young rats, which was not significantly affected by hydralazine (figure 3 A-E). Removal of the endothelium (figure 3A) and NO synthesis blockade (L-NAME, figure 3B) suppressed AT2R-dependent dilation in young rats, whereas the indomethacin did not affect AT2R-dependent dilation (figure 3C). AT2R blockade with PD 123319 abolished the dilation (figure 3D) and bradykinin B2 receptor blockade with HOE 140 (figure 3E) significantly decreased AT2R-dependent dilation in young rats, treated or not with hydralazine.
In old rats AT2R stimulation induced contraction (figure 3A-E). In old rats treated with hydralazine AT2R-dependent contraction was suppressed. Endothelium removal (figure 3A), NO synthesis blockade (L-NAME, figure 3B), cycloxygenase blockade with indomethacin (figure 3C) or bradykinin B2 receptor blockade with HOE 140 (figure 3E) did not affect AT2R-dependent contraction (figure 3A). AT2R blockade with PD 123319 abolished AT2R-induced contraction in old rats (figure 3D).

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Antioxydant treatment of the isolated MRA with tempol did not affect AT2R-dependent dilation in young rats but it suppressed AT2R-dependent contraction on old rats (figure 4A).
Reactive oxygen species were detected using DHE staining and confocal microscopy (figure 4B). In old rats, reactive oxygen species level was significantly higher in the endothelium and in the smooth muscle cell layer than in young animals (figure 4C). Hydralazine had no significant effect on ROS production in young rats arteries. In old rats, hydralazine significantly reduced ROS level in both endothelial and smooth muscle cells (figure 4C).

DISCUSSION

This study identified a paradoxical role of the angiotensin II type 2 receptor (AT2R) in resistance arteries isolated from old rats. Stimulation of AT2R by endogenous (in response to shear stress) or exogenous angiotensin II induced contraction through the production of reactive oxygen species (ROS) by smooth muscle cells in old rats. This effect contrasts with AT2R-dependent dilation in young rats. A treatment with hydralazine improved FMD in old rats through a strong reduction in ROS production.

Myogenic tone was lower in old than in young rats as previously shown in mesenteric and coronary arteries [17, 18]. Aging was also associated with a decreased FMD with a rightward shift of the flow rate response curve (lower sensitivity) and a decreased maximal response. This is in agreement with previous reports in large arteries [3, 19], in rat soleus feed arteries [20] and in the human brachial circulation [11]. In parallel, we found in old rats a decreased involvement of NO in FMD, suggesting a decreased eNOS activity as previously shown [20-22].

The presence of AT2R in the adult vasculature is now well recognized, although its role remains a matter of controversy [23-25]. Our previous works suggest that AT2R stimulation, which does not induce desensitization, might have a role in hypertensive patients treated with AT1R blockers [26]. Indeed, AT1R blockers induce AT2R overexpression and increase circulating angiotensin II [25].
We found that AT2R-dependent dilation in mesenteric resistance arteries (young rats) depended on the presence of the endothelium and was inhibited by NO-synthesis blockade and bradykinin B2 receptor blockade whereas cycloxygenase inhibition with indomethacin did not affect the dilation. These findings are in agreements with our previous reports in the same arterial bed [2, 13, 14, 26] and in

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other arteries [25]. The involvement of the bradykinin B2 receptor in AT2R-dependent dilation has been described in several vascular territories including the mesentery [27] and in the renal circulation [28]. Heterodimerisation of bradykinin B2 receptor and AT2R might occur [29] after AT2R stimulation in order to activate NO production in endothelial cells. This interaction between the 2 receptors might be efficient in the 2 directions as bradykinin B2 receptor dilation involved in FMD is reduced by AT2R blockade in the mouse carotid artery [30]. On the other hand, AT2R located on smooth muscle cells can downregulate Rho/Rho kinase activity, at least in conditions inducing AT2R upregulation such as chronic AT1R blockade in hypertensive rats [31].

A main new finding of the present study is that FMD was counteracted by AT2R-dependent contraction in old rats. This effect was mainly significant with low flow values. Stimulation of AT2R by exogenous angiotensin II also produced a vasoconstriction in old rats, which was not suppressed by endothelium removal. In old rats AT2R expression was higher than in young animals and AT2R were mainly located to the smooth muscle cells layer. Finally, AT2R-dependent contraction was suppressed by the antioxidant tempol. Thus in aging, AT2R located in smooth muscle layer induced ROS-dependent contraction.

We have previously observed AT2R-induced contraction in spontaneously hypertensive rats (SHRs), also in association with a preferential muscular location, although the expression level of AT2R is low in SHRs [2]. In SHRs AT2R function depends on blood pressure. AT2R-induced contraction occurs with high blood pressure and AT2R-induced dilation with normal blood pressure [2].

In addition, AT2R-dependent contraction in old rats resistance arteries was suppressed by the antioxidant tempol although unaffected by endothelium removal or indomethacin. Thus AT2R-dependent contraction was mediated by ROS. Angiotensin II-induced contraction through AT1R also involves ROS in pathological situations [32]. Thus, AT2R in aging, at least in mesenteric resistance arteries, might induce contraction through a similar mechanism. Aging was associated with a high basal ROS production in resistance arteries as shown in other cell types [33]. Further supporting the key role of ROS in AT2R-dependent contraction, we found that hydralazine, which reduced ROS level in arteries from old rats, also suppressed AT2R-induced contraction.

The probability of developing cardiovascular diseases requiring a treatment with AT1R blockers or Angiotensin Converting Enzyme Inhibitors increases with age. AT1R blockers induce AT2R over-expression and increase circulating angiotensin II [25]. Both effects should produce additional vasodilation and thus increase the protective effect of AT1R blockade. Nevertheless, this protective effect in patients treated with AT1R blockers may not occur in old patients unless AT2R-dependent

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contraction is reversed by vasodilator treatments in aging as it is in hypertension [2]. This is possible as hydralazine suppressed AT2R-related contraction in the present study.

The beneficial effect of hydralazine on FMD in old rats is the second new finding of the present study. First, in hydralazine-treated old rats, FMD was restored to control level in association with an increased NO-dependent tone. Second, hydralazine suppressed AT2R-dependent contraction in old rats. The possibility to restore FMD to control level in old rats is a key finding showing that, in resistance arteries the alteration is not irreversible. We have previously shown that a chronic treatment with hydralazine improves FMD in association with outward hypertrophic remodeling due to increased mesenteric blood flow [15]. This is associated with an increased eNOS expression in arteries from hydralazine treated rats [15].

In old rats outward remodeling also occurred (as shown by an increased passive arterial diameter) in hydralazine-treated rats. Nevertheless, eNOS expression did not increase in old rats treated with hydralazine, although NO-dependent tone (efficacy of L-NAME) increased. In addition, in old rats treated with hydralazine, FMD was restored to control level, despite an absence of effect on eNOS expression. Nevertheless, FMD was enhanced for two reasons. 1) AT2R-induced contraction was suppressed and 2) NO-biodisponibility was enhanced, as visualized by an increased efficiency of L-NAME in blocking FMD in old rats resistance arteries. In both cases, ROS production had a central role. First, AT2R-induced contraction depended on ROS production and in hydralazine-treated rats AT2R-induced contraction was absent in parallel with the strong decrease in ROS level. Thus, hydralazine most probably suppressed AT2R-induced contraction thanks to its anti-oxidant property. This observation is in agreement with previous studies demonstrating that hydralazine is anti-oxidant [34]. Second, FMD might also be improved due the increased bioavailability of NO found in arteries from hydralazine-treated old rats. Indeed, this effect might also be associated with the anti-oxidant property of hydralazine. We found that the level of ROS in mesenteric arteries was decreased after treatment with hydralazine in both endothelial and smooth muscle cells. This is in agreement with previous studies showing that a reduction in ROS production in response to flow, in diabetic animals, restores FMD to control level [35].

PERSPECTIVES

We identified an important change in AT2R function in aging as AT2R stimulated by flow (endogenous angiotensin II) or by exogenous angiotensin II induced a contraction involving the production of ROS. This contraction contributed to lower FMD. This alteration might affect the efficiency of the treatments used to fight vascular disorders in old patients. This observation is

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especially important as the occurrence and severity of vascular diseases is largely related to vascular aging [36]. In addition, hydralazine improved FMD in resistance arteries from old rats, showing that the alteration is reversible. Hydralazine decreased ROS level in both endothelial and smooth muscle cells, thus improving FMD. This effect was due to the suppression of AT2R-induced contraction and through an increase of the NO availability.

Interestingly, the positive effect of hydralazine on FMD in old rats is comparable to the effect of exercise training [37]. Both hydralazine and exercise increase blood flow and FMD. Thus the present finding also provides a possible explanation for the beneficial effect of exercise training on resistance arteries in the elderly. Nevertheless, exercise training is not always possible or safe in the older patients. In this situation hydralazine or other vasodilator treatment (to be tested) might be an alternative when improving endothelium-dependent tone is necessary.

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No conflict of interest to disclose.

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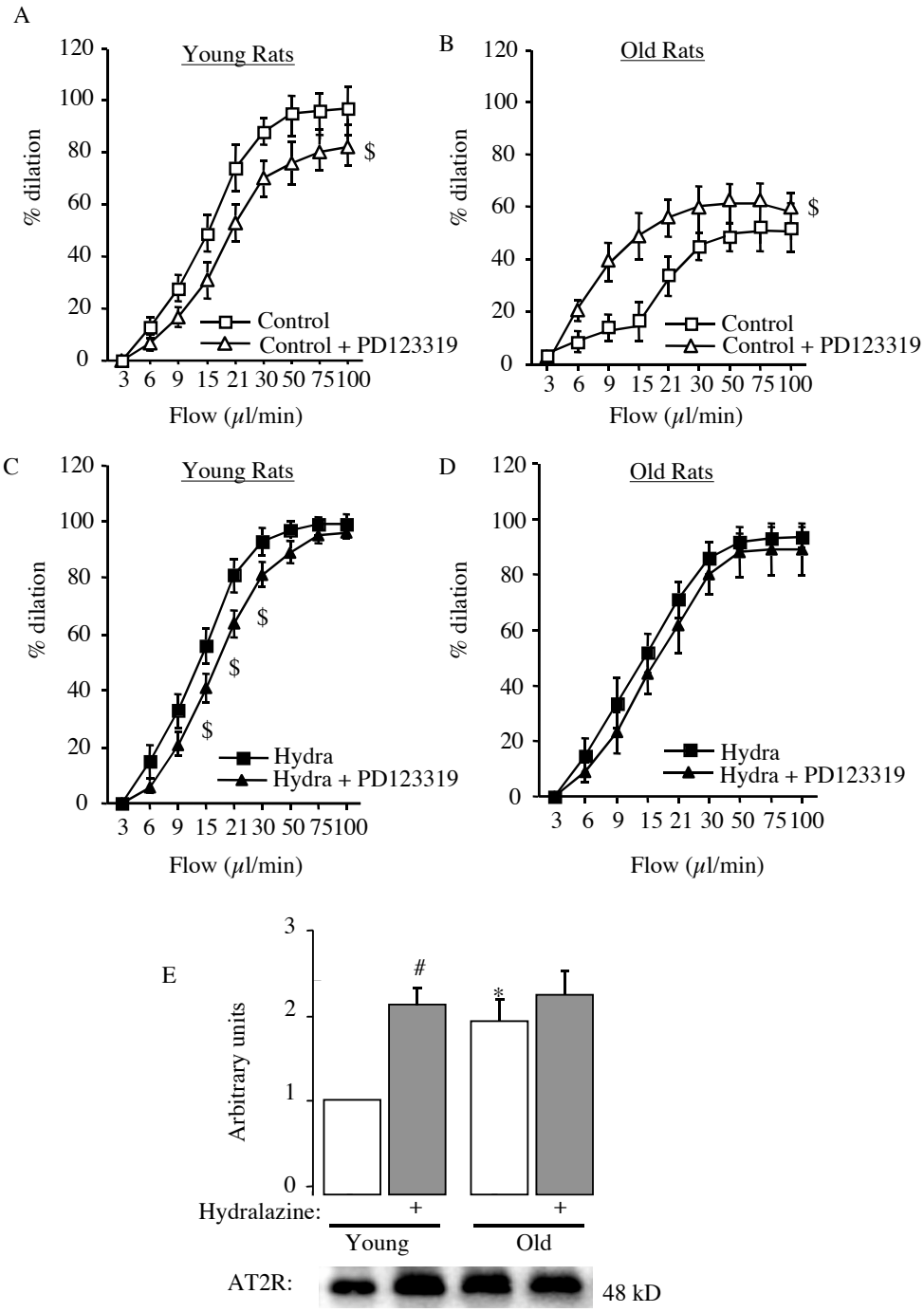


Figure 1: Effect of the AT2R antagonist PD123319 on flow-mediated dilation. Diameter changes in response to stepwise increases in flow in MRA were measured before and after addition of PD123319. Arteries were isolated from young (A & C) or old (B & D) rats treated with hydralazine (hydra, C & D) or water (control, A & B).
Panel E: Expression level of AT2R measured by western-blot analysis in MRA isolated from old or young rats treated with hydralazine (+) or water (-).
Mean ± sem is presented (n=12 old rats per group).
*P<0.01, old versus young rats.
#P<0.01, effect of hydralazine.
$P<0.01, effect of PD123319

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Figure 2

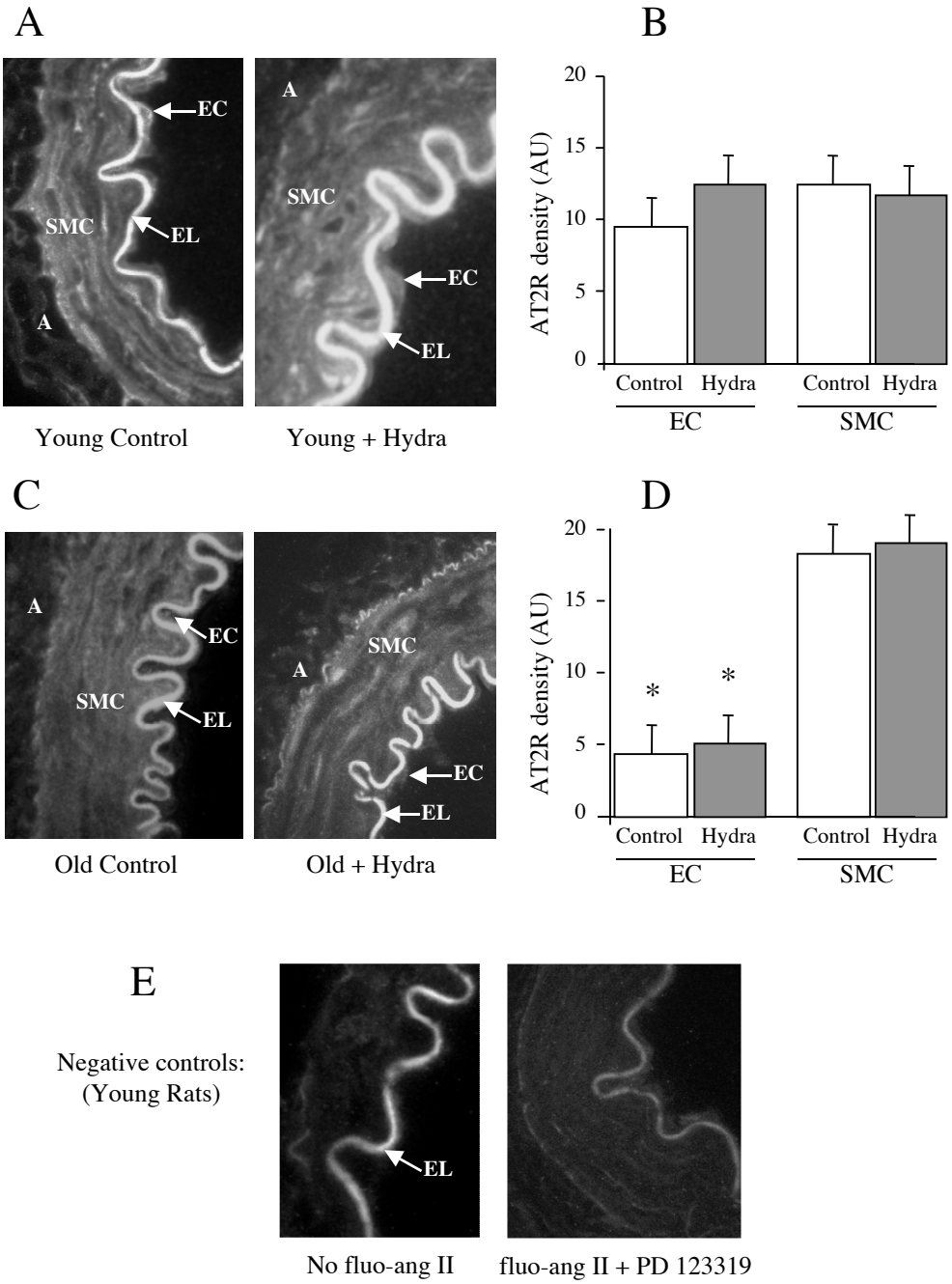
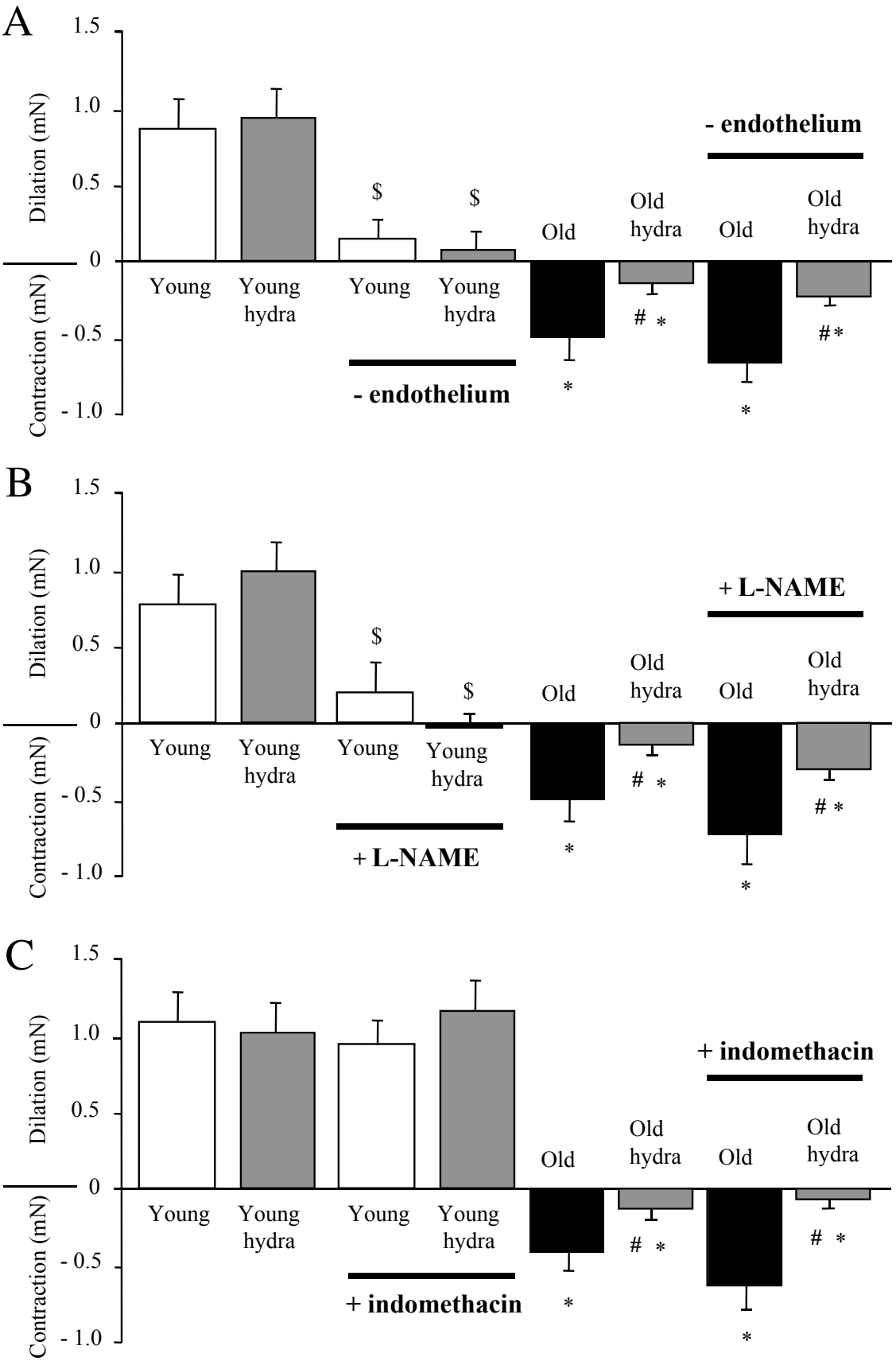


Figure 2: Localization of AT2R using fluorescent angiotensin II and confocal microscopy in MRA isolated from young (A & B) or old (C & D) rats treated with hydralazine (hydra) or water (control). (n=12 rats per group). Bargraphs (B & D) show the quantification of the AT2R in the endothelial cells (EC) layer and in the smooth muscle cells (SMC) layer. EL and A design the elastic lamina and the adventitia, respectively.
Panel E shows negative control experiments without fluorescent angiotensin II (fluo-angII) or with fluorescent angiotensin II plus the AT2R antagonist PD 123319.
*P<0.01, old versus young.

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Figure 3



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Figure 3

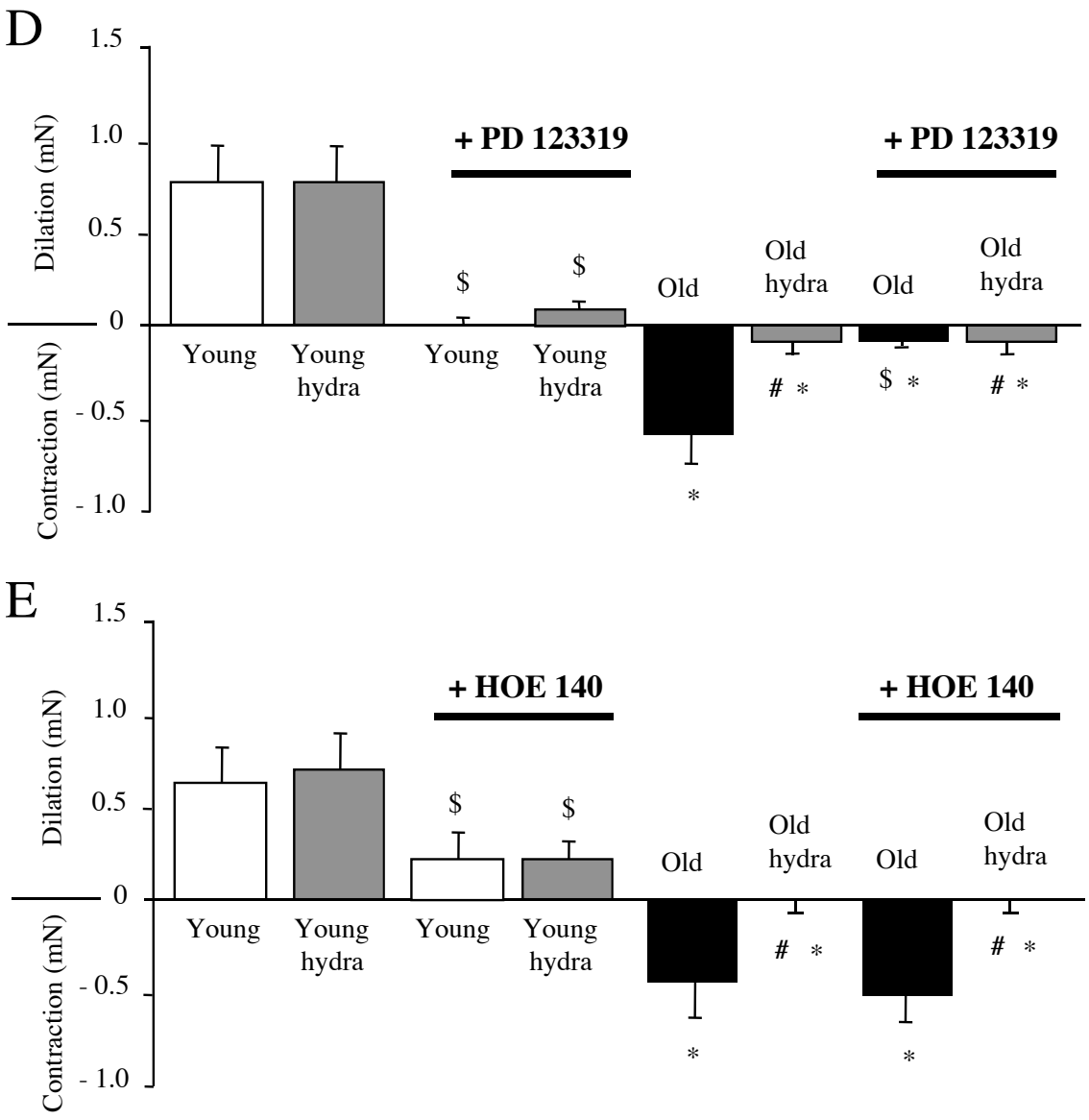


Figure 3: Contraction or dilation induced by angiotensin II measured in the presence of candesartan (AT2R-dependent effect) in MRA isolated from old or young rats treated with or without hydralazine (hydra). (n=12 rats per group).
A: Effect of AT2R stimulation with or without endothelium.
B: Effect of AT2R stimulation with or without NO synthesis blockade with L-NAME (100 µmol/L).
C: Effect of AT2R stimulation with or without cycloxygenase inhibition (indomethacin 10 µmol/L).
D: Effect of AT2R stimulation with or without AT2R blockade (PD 123319, 10 µmol/L).
E: Effect of AT2R stimulation with or without bradykinin B2 receptor blockade (HOE 140, 10 µmol/L).
*P<0.01, old rats versus the corresponding group in young rats.
#P<0.01, effect of hydralazine.
$P<0.01, effect of endothelium removal (A), L-NAME (B), indomethacin (C), PD 123319 (D) or HOE 140 (E).

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Figure 4

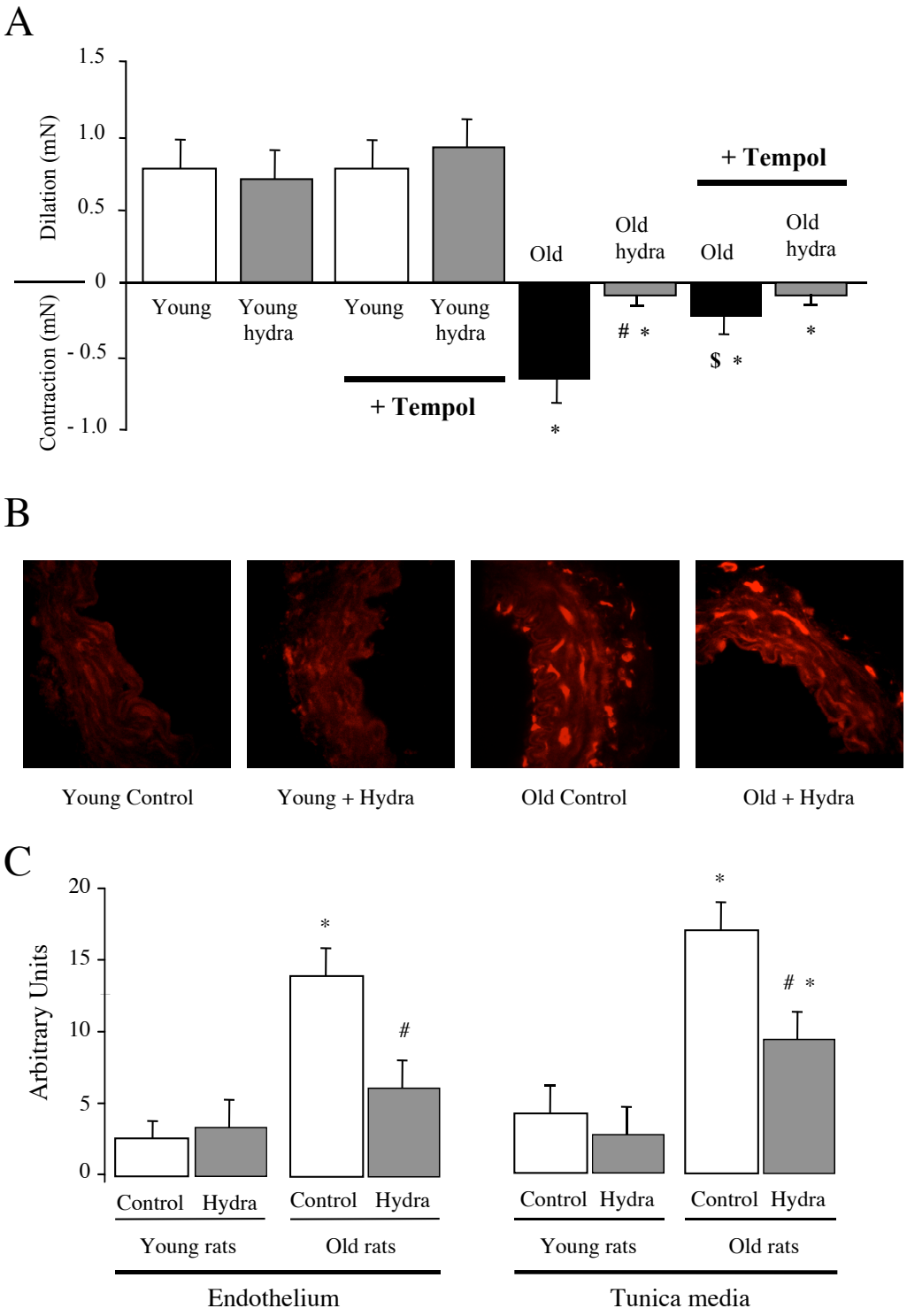


Figure 4: Effect of ROS reduction with tempol (**A**) on AT2R-dependent contraction or dilation in MRA isolated from old or young rats treated or not with or hydralazine (hydra). (n=12 rats per group). The level of reactive oxygen species (ROS) was measured in MRA isolated from old or young rats treated with hydralazine (hydra) or water (control): typical image obtained with confocal microscopy (**B**) and quantification of ROS in the endothelium and the tunica media (**C**). (n=12 old rats per group)
*P<0.01, old versus young rats.
#P<0.01, effect of hydralazine.
$P<0.01, effect of tempol (panel A).

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Supplement data

MATERIALS AND METHODS (SUPPLEMENT)

Pressure and flow-dependent tone in mesenteric resistance arteries

From each rat a segment of third order MRA, approximately 250 µm in internal diameter and 3-5 mm in length, was cannulated at both ends and mounted in a video monitored perfusion system as previously described [1]. Briefly, cannulated arterial segments were bathed in a 5 ml organ bath containing a physiological salt solution (PSS) of the following composition (in mmol/L): 135.0 NaCl, 15.0 $NaHCO_3$, 4.6 KCl, 1.5 $CaCl_2$, 1.2 $MgSO_4$, 11.0 glucose, 10.0 N-2-hydroxy-ethylpiperazine-N-2-ethylsulfonic acid. The pH was maintained at 7.4, pO_2 at 160 mmHg and pCO_2 at 37 mmHg. Perfusion of the arteries was performed with the same PSS. Arterial diameter was measured and recorded continuously using a video monitoring system (Living System Instrumentation Inc., Burlington, VT). Pressure and flow rate could be changed independently. In order to measure myogenic tone, equilibrium diameter changes were measured in each segment when intraluminal pressure was set at 10, 25, 50, 75, 100, 125 and 150 mmHg. Arteries were then submitted to 75 mmHg of pressure and further contracted of half the diameter with phenylephrine and serotonin in order to maintain a stable and reproducible tone in the different groups. Intraluminal flow was then increased by step (0 to 100 µl/min) and diameter measured. This was subsequently repeated after addition of the NO synthesis blocker NG-nitro-L-arginine methyl ester (L-NAME, 100 µmol/L) or the AT2R blocker PD123319 (10 µmol/L) to the PSS. At the end of each experiment arteries were bathed in a Ca^{2+}-free PSS containing ethylenbis-(oxyethylenenitrolo) tetra-acetic acid (EGTA, 2 mmol/L), sodium nitroprusside (10 µmol/L) and papaverine (10 µmol/L). Pressure steps (10 to 150 mmHg) were then repeated in order to determine the passive diameter of the vessel, i.e., in the absence of smooth muscle tone. Pressure and diameter measurements were collected using a Biopac data acquisition system (Biopac MP 100, La Jolla, CA, USA) and analyzed (Acqknowledge® software, Biopac). Myogenic tone was calculated as percentage of passive diameter and flow-mediated dilation as percentage dilation of active tone [1].

Before each experiment, the contractility of the muscle was tested using phenylephrine (1 µmol/L) and the integrity of the endothelium was assessed with acetylcholine (1 µmol/L) [1].

Vascular response to exogenous angiotensin II in isolated mesenteric arteries.

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Segments of mesenteric arteries (2 mm long) were mounted on a wire-myograph (DMT, Aarhus, DK) as previously described [2]. Briefly, 2 tungsten wires (25 µm diameter) were inserted in the lumen of the arteries and fixed to a force transducer and a micrometer, respectively. Arteries were bathed in a PSS maintained at 37°C, pH 7.4 (PO_2 160 mm Hg, PCO_2 37 mm Hg). The PSS contained candesartan (10^{-7} M) throughout the protocol in order to block AT1R. Wall tension was applied as described previously [3]. Vessels were then allowed to stabilize for one hour. Artery viability was tested using a potassium rich solution (80 mol/L, 80K PSS). They were then precontracted with phenylephrine (3 µmol/L) and serotonin (1 µmol/L) in order to obtain a sustained contraction. Angiotensin II (100 nmol/L) was then added to the bath. After washout, Angiotensin II (100 nmol/L) was again to the bath in the presence or absence of endothelium (effect of endothelium removal) or in the presence of endothelium plus one of the drugs cited above.

Western Blot Analysis of AT2 Receptors and eNOS expression

Western blot analysis of AT2R was performed in MRA as previously described [4]. Arterial segments (8 per group) were homogenized using a lysis buffer (1% sodium dodecyl sulfate, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L sodium orthovanadate, and proteases inhibitors cocktail). Extracts were incubated at 25°C for 30 minutes and then centrifuged (1000g, 15 minutes, 14°C). Proteins concentration was determined using the Micro BCA Protein Assay Kit (Pierce). After denaturation at 100°C for 5 minutes, equal amounts of proteins (15 µg) were loaded on a 9% polyacrylamide gel and transferred to nitrocellulose membranes for 90 min (100 V, 4°C). Membranes were blocked with 10% albumin bovine (BSA) in TBST (20 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, and 0.1% Tween-20) for 60 minutes and were then incubated with rabbit anti-AT2R polyclonal antibody (dilution 1/100, Santa Cruz, Califonia, USA) or with anti-eNOS mouse monoclonal antibody (dilution 1/1000, BD Transduction laboratories, USA) in BSA 5% in TBST overnight at 4°C. After extensive washing in TBST at room temperature, membranes were then incubated with the anti-rabbit horseradish peroxidase antibody (dilution 1/2000, Amersham Pharmacia Biotech, Orsay, France) or with the anti-mouse horseradish peroxidase antibody (dilution 1/1000) for 90 minutes at room temperature. After 3 washes with TBS-T, immunocomplexes were detected by chemiluminescent reaction (ECL-kit; Amersham Pharmacia Biotech) using a computer based imaging system (Fuji LAS 3000 plus; Fuji Medical System). Quantification was performed by densitometric analysis [4].

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Histological analysis of AT2 Receptors

Segments of MRA (n=12 old rats and 12 young rats) were mounted in embedding medium (Tissu-Tek, Miles, Inc), frozen in isopentane pre-cooled in liquid nitrogen, and stored at -80°C. Transverse cross sections (7 μm thick) were incubated with candesartan (30 min, 10 nmol/L, 25°C), then with fluorescent Angiotensin II (FITC-bound angiotensin II, 30 min, 10 pmol/L, 25°C, Molecular Probes) as previously described [4]. Fluorescence staining was visualized using confocal microscopy (Nikon, Eclipse TE2000S and Solamere Technology, Salt Lake City, UT, USA). Control experiments were performed after incubation with non-fluorescent Angiotensin II or in the presence of PD 123319. Image analysis was performed using Histolab (Microvision, France). Briefly, pixels quantification was performed after separating the media and the endothelial layer. Data is given as percentage of control [4].

RESULTS (SUPPLEMENT)

Rat body weight was not affected by hydralazine in young (310±10g in the presence of hydralazine, versus 318±9g) as well as in old rats (683±7g versus 696±12g).
Mean arterial blood pressure, measured in the femoral artery was not significantly different between control old rats (102±5 mmHg, n=12) and hydralazine-treated old rats (99±8 mmHg, n=12). Similarly, in young rats hydralazine did not significantly alter mean arterial blood pressure (104±5 mmHg in the presence of hydralazine versus 101±5 mmHg, n=12 per group).
Passive arterial diameter was higher in old than in young rats (figure I A and B). Hydralazine significantly increased passive and active arterial diameter in old rats (figure IA).
Isolated MRA submitted to stepwise increases in intraluminal pressure developed myogenic tone. Myogenic tone was lower in old than in young rats. Hydralazine had no significant effect on myogenic tone (figures I C).

Stepwise increases in flow (shear stress) induced a progressive vasodilation (figure ID). Flow-mediated dilation (FMD) was significantly lower in old than in young rats (figure ID). FMD was significantly higher in old rats treated with hydralazine than in control old rats. In young rats the effect of hydralazine, an increase in FMD, was significant for flow rates ranging between 6 and 21 μl/min (Bonferroni test), although a two-factor ANOVA analysis for repeated measures (flow

rates) did not show a significant difference on the whole curve (figure ID).

Phenylephrine induced a significant contraction in MRA, which was not significantly affected by aging or by hydralazine (figure IIA).
Acetylcholine-induced dilation in MRA was lower in old than in young rats. It was not significantly affected by hydralazine (figure IIB).

NO synthesis blockade with L-NAME reduced FMD in all groups (figure III A & B). The effect of L-NAME was lower in old than in young rats (figure IIIB). In hydralazine-treated rats, the effect of L-NAME was significantly higher than in untreated rats (figure 3A and B).
Western blot analysis performed in MRA showed that eNOS expression was lower in old than in young rats. In young rats, hydralazine significantly increased eNOS expression (figure IIIC).

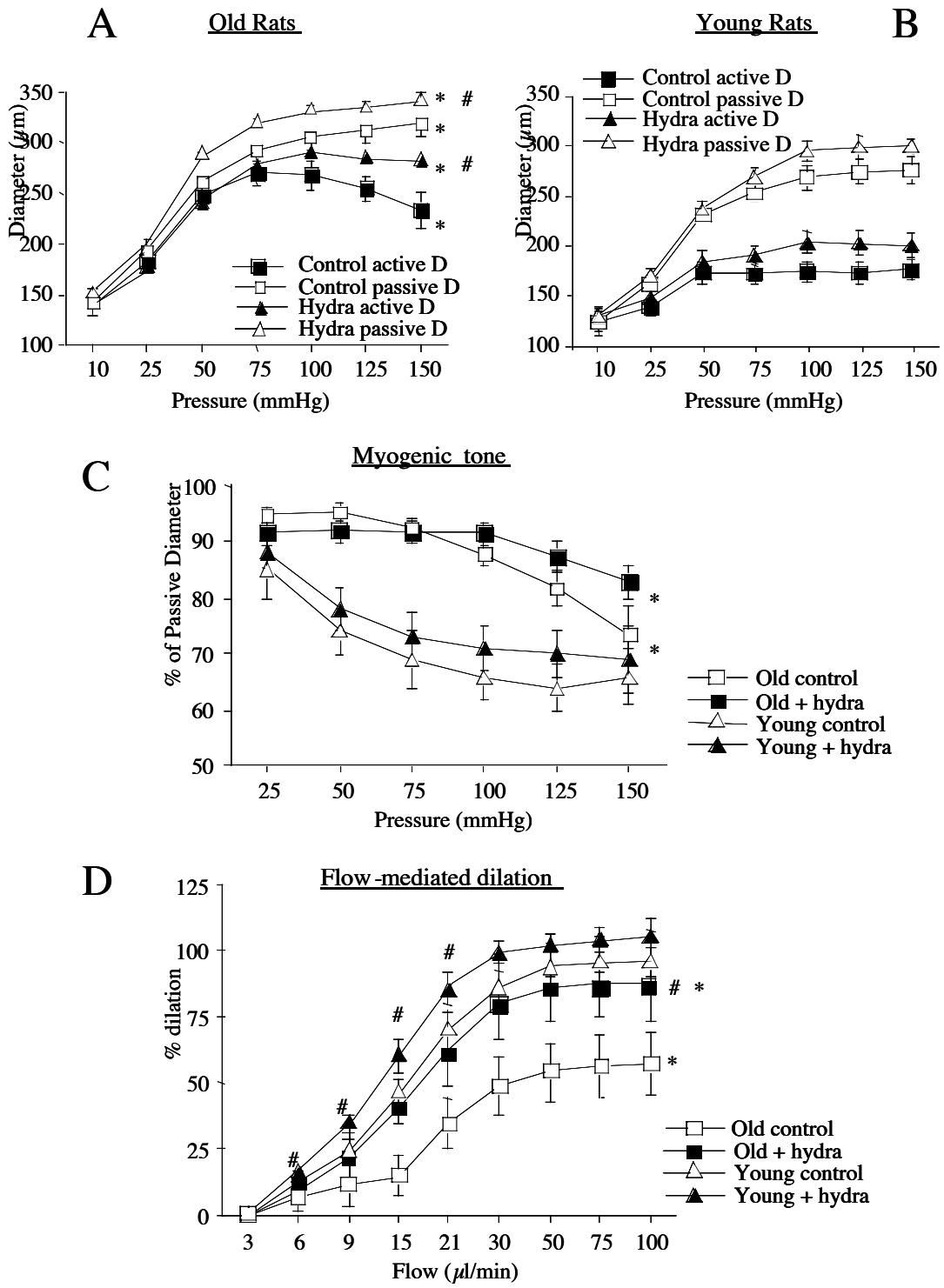
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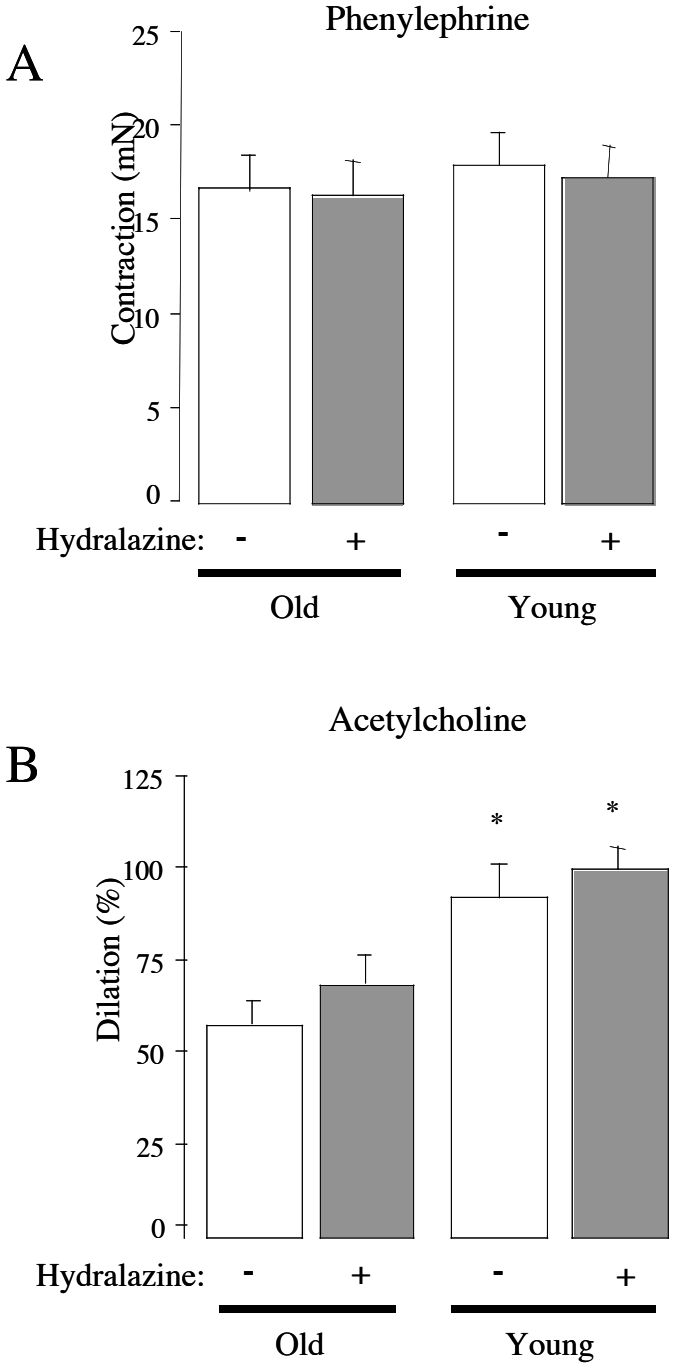
Figure I



A & B: Diameter changes in response to stepwise increases in pressure in MRA isolated from old (A) or young (B) rats treated with hydralazine (hydra) or water (control). Active and passive diameters are shown (A & B) and were used to determine myogenic tone (C: active diameter expressed as percentage of passive diameter).
D: Diameter changes in response to stepwise increases in flow in MRA isolated from old and young rats treated with hydralazine (hydra) or water (control).
Mean ± sem is presented (n=12 old rats per group).
*P<0.01, old versus young rats
#P<0.01, effect of hydralazine.

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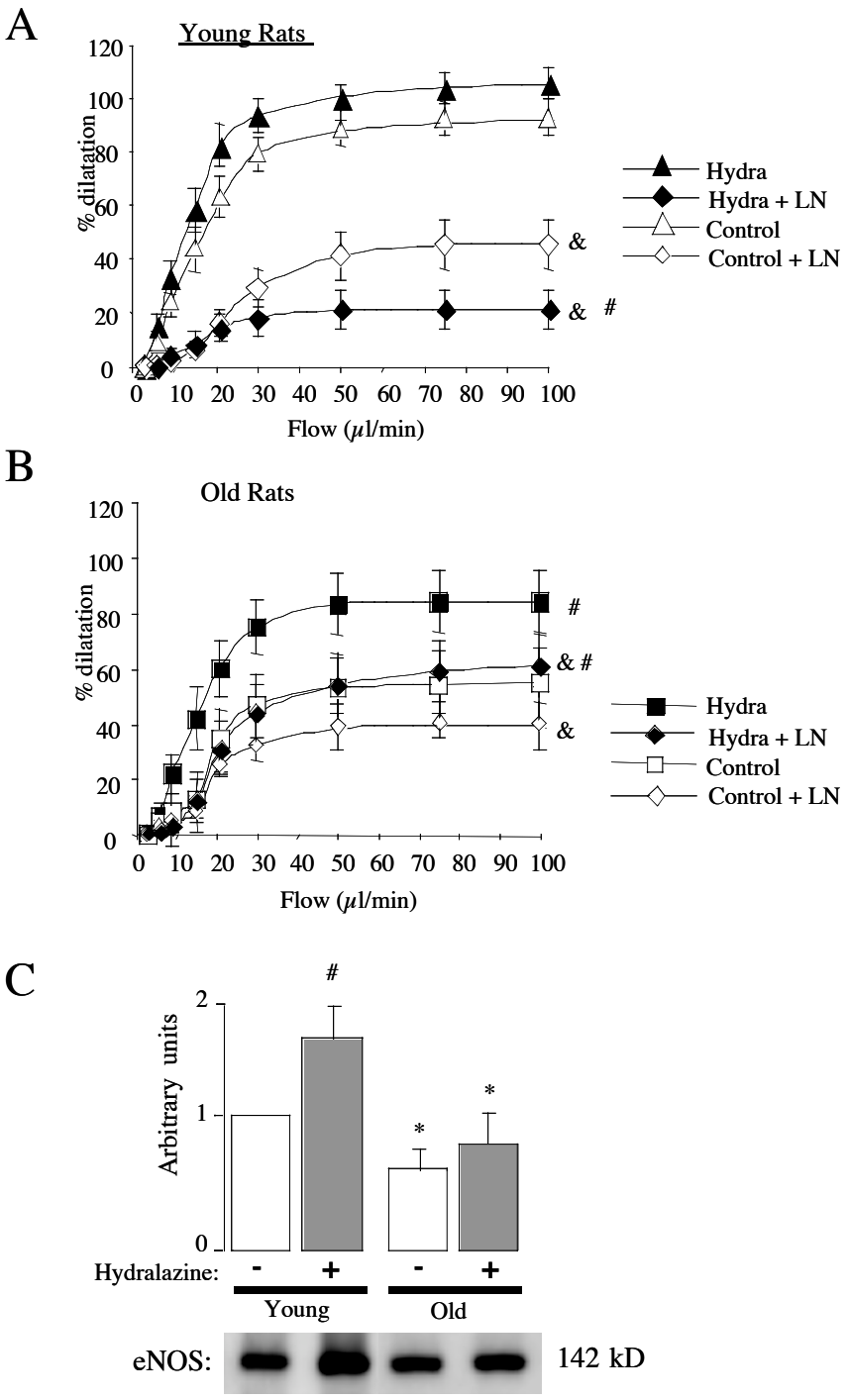
Figure II



Contraction induced by phenylephrine (1 µmol/L, **A**) and vasorelaxation induced by acetylcholine (1 µmol/L, **B**) after precontraction with phenylephrine in MRA isolated from old and young rats treated with hydralazine (+) or water (-) (n=12 old rats per group).
*P<0.01, old versus young rats
No significant effect of hydralazine.

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Figure III



Effect of NO synthesis blockade with L-NAME (LN, 100 µmol/L) on flow-mediated dilation in MRA isolated from young (**A**) and old (**B**) old rats treated with hydralazine (hydra) or water (control).
Panel C: expression level of eNOS measured by western-blot analysis in MRA isolated from old or young rats treated with hydralazine (+) or water (-).
Mean ± sem is presented (n=12 old rats per group).
*P<0.01, old versus young rats.