

## Effect of Age on Kinetics of Nitric Oxide Release in Rat Aorta and Pulmonary Artery

Marcel Roger Tschudi,\* Matthias Barton,\* Nick Arnold Bersinger,† Pierre Moreau,\* Francesco Cosentino,\* Georg Noll,\* Tadeusz Malinski,§ and Thomas Felix Lüscher\*

\*Cardiovascular Research, Cardiology, University Hospital Bern, CH-3010 Bern, Switzerland; †Clinical Hospital for Obstetrics and Gynaecology, CH-3010 Bern, Switzerland; and §Department of Chemistry, Institute of Biotechnology, Oakland University, Rochester, Michigan 48063

### Abstract

Aging is an important determinant of vascular disease. Endothelium-derived nitric oxide (NO) is protective as a vasodilator and inhibitor of platelet function. This study was designed to directly measure effects of prolonged aging on endothelial NO release in isolated blood vessels and to delineate differences between the systemic and pulmonary circulation.

Aortas and pulmonary arteries from 5–6-mo-old (young), 18–19-mo-old (middle-aged), and 32–33-mo-old (old) normotensive female rats were used. Blood pressure and plasma estradiol-17 $\beta$  (E<sub>2</sub>) remained unchanged. In isolated blood vessels, NO release was induced by the receptor-independent agonist calcium ionophore A23187 (10  $\mu$ mol/liter) and measured in situ on the endothelial surface of vessels using a porphyrinic microsensor. In vessels suspended in organ chambers isometric tension was recorded. In the aorta, the initial rate of NO release and peak NO concentration were reduced in middle-aged and old rats ( $P < 0.0006$  vs. young rats,  $n = 6$ ). Furthermore, endothelium-dependent relaxations to calcium ionophore and acetylcholine (both  $10^{-10}$ – $10^{-5}$  mol/liter) were also reduced in aortas from old as compared with young rats ( $n = 6$ ,  $P < 0.05$ ). The initial rate of NO release and peak NO concentration significantly correlated with maximal relaxation to calcium ionophore A23187 (correlation coefficients  $r = 0.916$ ,  $P < 0.0018$  and  $r = 0.961$ ,  $P < 0.0001$ , respectively,  $n = 7$ ). In pulmonary arteries, however, the initial rate of NO release as well as peak NO concentration did not decrease with age ( $n = 6$  for each age group, NS). In both blood vessels, the NO release was unaffected by superoxide dismutase in all age groups ( $n = 6$ , NS).

Thus, aging specifically reduces initial rate and peak concentrations of endothelial NO release from aorta but not pulmonary artery indicating reduced NO production. As ar-

terial pressure did not change with aging, the chronic exposure of the aorta to higher pressure and/or pulsatility than in the pulmonary artery may be the cause. This appears important as NO plays a protective role by preventing vasoconstriction, thrombosis and atherosclerosis. (*J. Clin. Invest.* 1996; 98:899–905.) Key words: acetylcholine • calcium ionophore A23187 • Endothelium-derived nitric oxide • porphyrinic NO microsensor • vasorelaxation

### Introduction

The endothelium contributes to the control of vascular smooth muscle tone by the release of nitric oxide (NO), which accounts for the biological activity of the endothelium-derived relaxing factor (1–4). NO causes vasodilatation and platelet inhibition and thereby prevents vasoconstriction and thrombus formation (5). NO is generated from a terminal guanidino nitrogen of L-arginine and catalyzed by a family of enzymes called NO synthases (6, 7). One of these enzymes, i.e., eNOS, is Ca<sup>2+</sup>-dependent and constitutively present in various types of cells, including endothelial cells (8).

The short half-life of NO has created severe problems in its direct determination. Electrochemical methods permit direct in situ measurement of NO in biological samples (9). The porphyrinic microsensor also is based on the electrochemical oxidation of NO and measurement of a current generated in this process (4, 10). The high sensitivity and small size of the porphyrinic NO sensor are favorable features that permit in situ monitoring of NO on the endothelial surface of isolated blood vessels (11, 12). However, the most significant advantage of the porphyrinic microsensor is that its fast response time (0.1–10 ms) makes it ideal for measurements of the kinetics of NO release (13, 14).

All forms of cardiovascular disease increase in frequency with age, even in the absence of cardiovascular risk factors (5). This suggests that aging per se alters vascular function. Intimal proliferation occurs in pulmonary arteries from infancy with changes similar to, but less severe than in the aorta (15). Aging also exerts functional changes of endothelial cells. Several studies indicate that endothelium-dependent relaxations might decline with age (16–19). In these studies, however, NO release has not been measured directly and only judged from its physiological effects (i.e., relaxation of blood vessels). This, however, also involves other endothelium-derived relaxing factors than NO (20). In addition, all studies have investigated middle-aged (1.5 or 2 yr) rather than old animals. Hence, so far no reliable data on the kinetics and amount of NO released from blood vessels of rats with a very high age of 2.5 yr and be-

Address correspondence to Thomas F. Lüscher, MD, Professor of Medicine, Division of Cardiology, University Hospital Inselspital, CH-3010 Bern, Switzerland. Phone: 31 632 96 52; FAX: 31 382 10 69.

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yond are available. As aging may at least in part depend on hemodynamic factors such as pressure and pulsatility, it was further the aim of the present study to compare the effects of prolonged aging on the kinetics of NO release in the aorta and pulmonary artery of the rat.

## Methods

### Surgical procedures

Female normotensive RORO rats, 5–6 mo old (young), 18–19 mo old (middle), and 32–33 mo old (old) were obtained from Biological Research Laboratories Ltd. (Füllinsdorf, Switzerland). On the day of the experiment rats were anesthetized with thiopental (50 mg/kg body weight i.p.) and blood pressure was measured in the left femoral artery with a Letica PRI 256/2 equipment (Letica S/A Instruments, Hospitalet, Spain). The rats were then decapitated and the chest and abdomen were opened through a medial sternotomy. Subsequently, the aorta and pulmonary artery were excised and immediately placed in cold (4°C) modified Hank's balanced salt solution (HBSS) of the following concentration (in mmol/liter): NaCl, 137; Tris-HCl, 10; MgCl<sub>2</sub>, 1; KCl, 5; CaCl<sub>2</sub>, 0.9; MgSO<sub>4</sub>, 0.8; KH<sub>2</sub>PO<sub>4</sub>, 0.44; Na<sub>2</sub>HPO<sub>4</sub>, 0.33; L-arginine, 0.1 (pH = 7.40). Under a dissection microscope (Wild M3C; Wild AG, Heerbrugg, Switzerland) isolated blood vessels were cleaned of adhering tissue and cut into rings of 5 mm in length. All these procedures were approved by the Commission for Animal Research of the Canton of Bern, Switzerland.

### Determination of plasma estradiol-17β (E<sub>2</sub>)

Estradiol-17β (E<sub>2</sub>) was determined using a time-resolved immunofluorimetric method (DELFLIA) with a microplate kit (Wallac, Finland). For extraction, 80 μl of serum were mixed with 1 ml of diethyl ether in a polypropylene tube. After vortexing, the tube was immersed in liquid nitrogen and, after solidification of the aqueous phase at the bottom, the organic phase was immediately and quantitatively decanted into a 2-ml Eppendorf tube and evaporated using a speed-Vac concentrator with trap (Savant Instruments, Inc., Farmingdale, NY). The sample was recovered with an identical volume of DELFLIA assay buffer. The E<sub>2</sub> assay itself was run according to the manufacturer's instructions in a single competitive incubation. The serum-based standards from the kit were run nontreated as well as after extraction; from this comparison we calculated an extraction efficiency of 71% (range 65–79%). Direct (nonextracted human serum) intra-assay coefficients of variance (CV) were 10% at 0.1 nmol/liter and 5% at 1 nmol/liter according to the assay manual; after duplicate extraction we found cumulative intra-assay CV's of 19 and 7%, respectively.

### Porphyritic NO microsensor

**NO microsensor fabrication.** Measurements of NO were carried out with a porphyritic microsensor (4). The NO microsensor was produced by threading an array of 5 to 10 carbon fibers (AMOCO Performance Products, Inc., Geneva, Switzerland) through a pulled end of an L-shape glass capillary with 5 mm length of the fibers left protruding. A copper contact wire was inserted in the opposite end of the glass capillary and sealed with conductive silver epoxy (A.I. Technology, Princeton, NJ). Then the tip of the glass capillary was sealed with bee's wax. A conductive polymeric film was then deposited on the surface of the carbon fibers from a 0.25 mM solution of nickel (II) tetrakis (3-methoxy-4-hydroxyphenyl) porphyrin in 0.1 M NaOH under N<sub>2</sub> as previously described (4, 10). After drying, the sensor's active tip was immersed in 1% (wt) Nafion solution in alcohol (Aldrich Chemie, Buchs, Switzerland) for 15 s and then allowed to dry again.

**Experimental setup.** A three-electrode system was used for the NO measurements, consisting of the NO sensor working electrode, a standard calomel reference electrode, and a platinum wire auxiliary electrode. Measurements of NO were carried out with an EG&G PAR Model 264A voltametric analyzer (EG&G GmbH, München,

Germany). Amperometric mode detection was used at a constant potential equal to the peak potential for NO oxidation of the electrode and modulated with 50 mV pulse in time intervals of 0.5 s. The amperometric signal was recorded with a Kipp & Zonen flatbed chart recorder BD-112 (Recom Electronic AG, Horgen-Arn, Switzerland) and NO concentration was determined from the measured current by means of a calibration curve with NO standards. Standard NO solutions (1.8 mmol/liter) were prepared from aqueous solution saturated with pure gaseous NO (Garbagas, Liebefeld, Switzerland).

**Protocols.** Immediately before NO measurements, isolated vascular ring-segments were cut longitudinally and pinned on the bottom of an organ chamber filled with fresh HBSS buffer (5 ml, 37°C, pH = 7.40). Then the active tip (length, 5 mm; diameter, 20–30 μm) of the L-shape NO microsensor was placed on the endothelial surface of the aortic or pulmonary artery strips. A precision stereo zoom microscope PZM and a micromanipulator M3301 (both from World Precision Instruments, Berlin, Germany) were used for the microsensor positioning. Then 10 μl of a 10 μmol/liter calcium ionophore A23187 solution (maximal stimulation of the NO synthase) was injected on the luminal side of isolated vascular strips with a pneumatic picoinjector PV820 (World Precision Instruments, Berlin, Germany) positioned with a micromanipulator. Experiments were then repeated in the presence of superoxide dismutase (SOD; 100 U/ml).

In separate experiments, vessels were preincubated with N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 2 × 10<sup>-4</sup> mol/liter) for 30 min before NO measurements.

### Organ chambers

**Experimental setup.** To study endothelium-dependent relaxations, aortic rings were mounted horizontally between two stirrups in organ chambers filled with 25 ml Krebs-Ringer bicarbonate solution (37°C, 95% O<sub>2</sub>, 5% CO<sub>2</sub>) of the following composition (mmol/liter): NaCl, 118.6; KCl, 4.8; CaCl<sub>2</sub>, 2.5; MgSO<sub>4</sub>, 1.2; KH<sub>2</sub>PO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 25.1; edetate calcium disodium, 0.026; glucose, 10.1. One stirrup was connected to an anchor and the other to a force transducer (UTC2, Gould Statham, UK) for recording of isometric tension. After a 30-min equilibration period, rings were progressively stretched until the contractile response to high potassium chloride/Krebs-Ringer solution (composition in mmol/liter: NaCl, 23.4; KCl, 100.0; CaCl<sub>2</sub>, 2.5; MgSO<sub>4</sub>, 1.2; KH<sub>2</sub>PO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 25.1; edetate calcium disodium, 0.026; glucose, 10.1) was maximal. The aortic rings were allowed to equilibrate for 30 min before the experiments.

**Protocols.** For endothelium-dependent relaxations, vessels were contracted with 2 × 10<sup>-7</sup> mol/liter norepinephrine, and then relaxed with 10<sup>-10</sup>–10<sup>-5</sup> mol/liter calcium ionophore A23187 or acetylcholine. In terms of percent of maximal contraction to KCl 100 mmol/liter, the precontraction levels in response to 2 × 10<sup>-7</sup> mol/liter norepinephrine were comparable in young and old rats (*P* > 0.2).

**Table I. Diastolic Blood Pressure, Systolic Blood Pressure, Pulse Pressure, Mean Blood Pressure, Body Weight, and Plasma Estradiol-17β of Young (5–6-mo-old; n = 6–7), Middle Aged (18–19-mo-old; n = 6–7) and Old (32–33-mo-old; n = 6–7) RORO Rats**

Parameter	Young	Middle	Old
Diastolic blood pressure (mm Hg)	78±3	96±9	82±7
Systolic blood pressure (mm Hg)	98±3	119±10	112±8
Pulse pressure (mm Hg)	20±1	22±2	30±3*
Mean blood pressure (mm Hg)	84±3	104±9	92±7
Body weight (g)	222±5	316±5*	294±4*‡
Plasma estradiol-17β (nmol/liter)	0.13±0.07	0.19±0.09	0.12±0.08

Data are given as mean±SEM. \**P* < 0.05; vs Young, ‡*P* < 0.05; vs Middle.

## Drugs

Calcium ionophore A23187, acetylcholine chloride, superoxide dismutase (SOD), N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) and chemical components of the physiological salt solutions (HBSS and Krebs-Ringer) were obtained from Sigma Chemical Company (Buchs, Switzerland).

## Calculations and statistical analysis

For statistical analysis, the initial rate of NO release (slope of NO peak; nmol/liter/s), the maximal concentration of NO produced (NO peak height; nmol/liter) and the maximal vasorelaxation (expressed as percentage of a previous contraction to  $2 \times 10^{-7}$  mol/liter norepinephrine) was measured in isolated blood vessels. Data are given as means  $\pm$  SEM. In each set of experiments,  $n$  is the number of animals studied. Statistical analysis was done by unpaired Student's  $t$  test or by ANOVA followed by Scheffé's  $F$  test. In addition, the correlation coefficient ( $r$ ) between endothelial NO release and maximal relaxation was calculated and statistical analysis was done by Fisher's  $r$  to  $z$  test. Means were considered significantly different at  $P < 0.05$ .

## Results

### Blood pressure, body weight and plasma estradiol-17 $\beta$

Diastolic blood pressure ( $n = 6$ ), systolic blood pressure ( $n = 6$ ), pulse pressure ( $n = 6$ ), mean blood pressure ( $n = 6$ ), body weight ( $n = 6$ ) and plasma estradiol-17 $\beta$  (E<sub>2</sub>;  $n = 7$ ) of young (5–6-mo-old), middle-aged (18–19-mo-old), and old (32–33-mo-old) female RORO rats are given in Table I.

### Endothelial NO release in aortas

*Direct in situ measurements of NO.* Typical amperometric (current proportional to concentration vs. time) curves obtained for in situ ex vivo measurements of NO in an aorta of a young and old normotensive RORO rat are presented in the upper panels of Fig. 1. A rapid release of NO was observed after injection of 10  $\mu$ l of a 10  $\mu$ mol/liter calcium ionophore A23187 solution with a microinjector in close proximity to the L-shaped

porphyrinic NO sensor placed on the endothelial surface of rat aortas. The initial rate of NO release (nmol/liter/s) as well as the peak NO concentration (maximal endothelial NO surface concentration; nmol/liter) were significantly reduced in the aorta obtained from an old rat when compared with the one obtained from a young rat.

Fig. 2 presents typical amperograms showing NO release in the aorta of a middle-aged RORO rat in presence (right panel) and absence (left panel) of the inhibitor of NO production N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME). NO release was almost completely blocked after incubation of isolated aortic strips with L-NAME ( $2 \times 10^{-4}$  mol/liter) for 30 min.

Fig. 3 (left) summarizes data from aortas obtained from six young, six middle-aged, and six old RORO rats. The initial rate of NO release after calcium ionophore administration (10  $\mu$ mol/liter) was significantly slower in aortas from middle-aged rats and old rats when compared with aortas obtained from young rats (young:  $619 \pm 43$  nmol/liter/s; middle-aged:  $241 \pm 43$  nmol/liter/s,  $P < 0.0001$  vs. young; old:  $237 \pm 32$  nmol/liter/s,  $P < 0.0001$  vs. young). There was a statistically significant correlation between the initial rate of NO release and the maximal relaxation after calcium ionophore A23187 ( $10^{-5}$  mol/liter) administration (correlation coefficient  $r = 0.916$ ,  $P < 0.0018$ ,  $n = 7$ ).

Fig. 4 (left) presents the average peak NO concentrations in rat aortas following stimulation with calcium ionophore A23187 (10  $\mu$ mol/liter). The maximal NO levels measured by the porphyrinic microsensor were significantly lower in aortas obtained from middle-aged rats and old rats when compared with aortas from young rats (young:  $1504 \pm 146$  nmol/liter; middle-aged:  $616 \pm 124$  nmol/liter,  $P < 0.0006$  vs. young; old:  $536 \pm 102$  nmol/liter,  $P < 0.0003$  vs. young,  $n = 6$  for each age group). The correlation between NO peak concentration and maximal relaxation after calcium ionophore A23187 administration ( $10^{-6}$  mol/liter) was highly significant (correlation coefficient  $r = 0.961$ ,  $P < 0.0001$ ).

In the aorta, the initial rate of NO release as well as peak

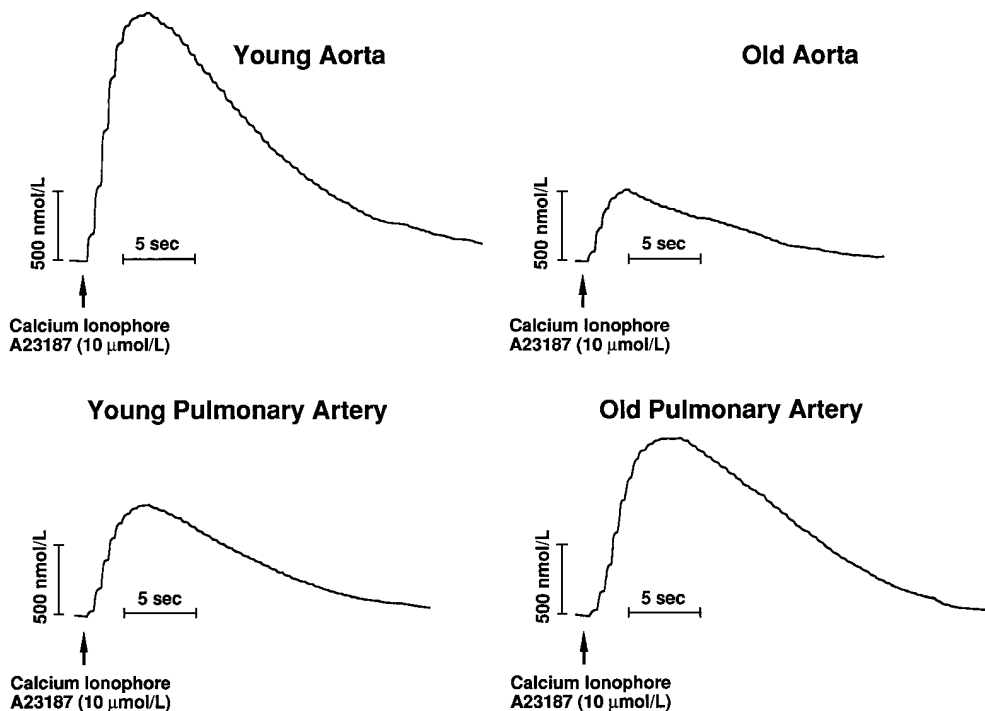
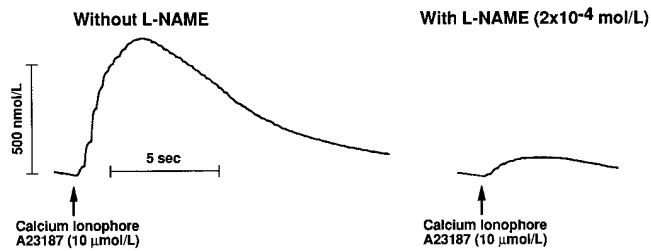


Figure 1. Amperograms of nitric oxide release from isolated aortas (upper panels) and pulmonary arteries (lower panels) of young (8–9 mo old) and old (32–33 mo old) normotensive RORO rats. The nitric oxide release was agonized by calcium ionophore A23187 (10  $\mu$ mol/liter) and measured in situ on the endothelial surface of the vascular strips using a porphyrinic microsensor. With age nitric oxide release significantly decreased in aortas while mildly increased in pulmonary arteries.



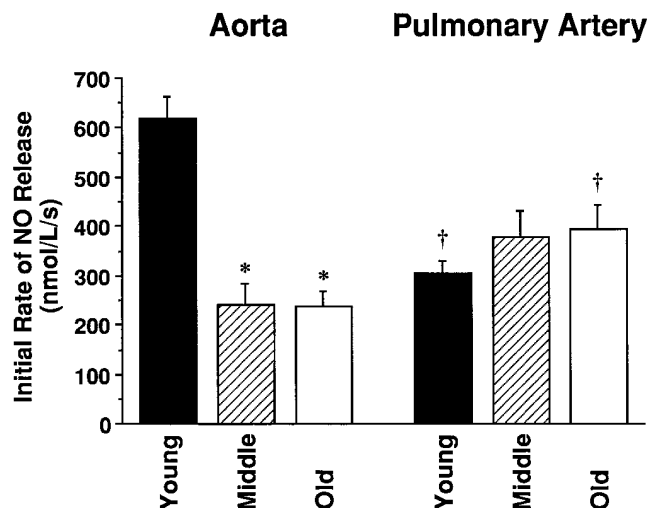
**Figure 2.** Typical amperograms showing NO release after calcium ionophore A23187 administration (10  $\mu\text{mol/liter}$ ) in the aorta of a middle-aged RORO rat (18–19 mo old) in presence (*right panel*) and absence (*left panel*) of the inhibitor of NO production N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME). NO release was almost completely blocked after incubation of isolated aortic strips with L-NAME (2  $\times 10^{-4}$  mol/liter) for 30 min.

NO concentration were unaffected by the superoxide scavenger superoxide dismutase (100 U/ml; Table II).

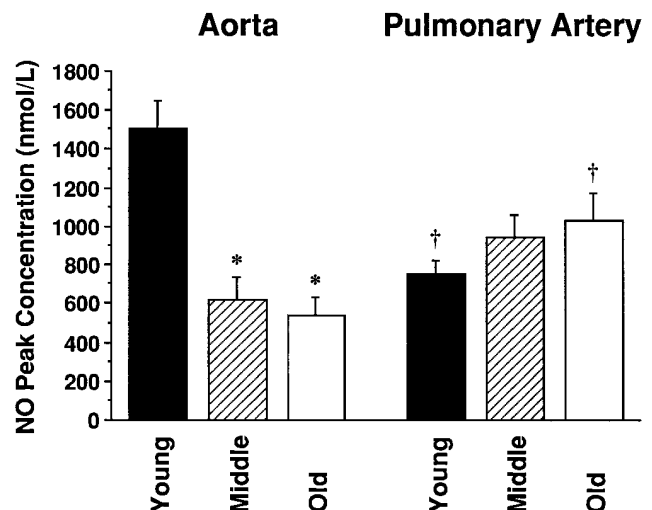
**Endothelium-dependent relaxations.** Aortas from old rats exhibited reduced relaxations to the receptor-independent agonist calcium-ionophore A23187 ( $10^{-10}$ – $10^{-5}$  mol/liter, Fig. 5, *left panel*) and the receptor-dependent agonist acetylcholine ( $10^{-10}$ – $10^{-5}$  mol/liter, Fig. 5, *right panel*) as compared with those of young rats ( $P < 0.05$  for  $3 \times 10^{-9}$  to  $10^{-5}$  mol/liter,  $n = 6$ ).

#### Endothelial NO release in pulmonary arteries

**Direct in situ measurements of NO.** Typical amperograms (current-concentration vs. time) showing NO release in a pulmonary artery obtained from a young and old normotensive RORO rat are depicted in the lower panels of Fig. 1. Immediately after calcium ionophore A23187 administration (10  $\mu\text{mol/liter}$ ), an initial rapid increase of NO concentration was observed. The velocity of NO release (nmol/liter/s) and the



**Figure 3.** Bar graphs showing the initial rate of NO release measured in situ on the endothelial surface of isolated aortas and pulmonary arteries of normotensive RORO rats at different ages (young: 5–6 mo old; middle: 18–19 mo old; and old: 32–33 mo old). The nitric oxide release was agonized by the receptor-independent agonist calcium ionophore A23187 (10  $\mu\text{mol/liter}$ ) and monitored on line using a porphyrinic microsensor. Values are means  $\pm$  SEM. \* $P < 0.0001$  vs. young aorta,  $\dagger P < 0.03$  vs. age matched aorta,  $n = 6$  for each age group.



**Figure 4.** Bar graphs showing maximal concentration of nitric oxide measured in situ on the endothelial surface of isolated aortas and pulmonary arteries of normotensive RORO rats at different ages (young: 5–6 mo old, middle: 18–19 mo old, and old: 32–33 mo old). The nitric oxide release was agonized by calcium ionophore A23187 (10  $\mu\text{mol/liter}$ ) and the maximal concentration of nitric oxide produced was measured using the porphyrinic microsensor. Values are means  $\pm$  SEM. \* $P < 0.0006$  vs. young aorta,  $\dagger P < 0.02$  vs. age matched aorta,  $n = 6$  for each age group.

maximal concentration of NO produced (peak NO concentration; nmol/liter) were considerably but not significantly increased in the pulmonary artery from the old rat when compared with the one obtained from a young rat.

Fig. 3 (*right*) summarizes data from pulmonary arteries obtained from six young, six middle-aged, and six old RORO rats. The initial rate of NO release after calcium ionophore administration (10  $\mu\text{mol/liter}$ ) was maintained and even increased from  $305 \pm 25$  nmol/liter/s in young rats, to  $380 \pm 50$  nmol/liter/s in middle-aged rats, and  $396 \pm 48$  nmol/liter/s in old rats ( $n = 6$  for each age group, NS).

The initial rate of NO release was significantly slower in pulmonary arteries from young rats when compared with aor-

**Table II.** The Average Rate of Nitric Oxide Concentration Increase and Peak Nitric Oxide Concentration in Aortas and Pulmonary Arteries of Young (5–6-mo-old;  $n = 6$ ), Middle Aged (18–19-mo-old;  $n = 6$ ) and Old (32–33-mo-old;  $n = 6$ ) Rats in Presence of the Superoxide Scavenger Superoxide Dismutase (100 U/ml)

Parameter	Young	Middle	Old
Initial rate of NO release in aorta	574 $\pm$ 31	285 $\pm$ 50*	211 $\pm$ 40*
Peak NO concentration in aorta	1364 $\pm$ 108	704 $\pm$ 164*	468 $\pm$ 91*
Initial rate of NO release in art. pul.	258 $\pm$ 46 <sup>‡</sup>	378 $\pm$ 24	436 $\pm$ 63 <sup>‡</sup>
Peak NO concentration in art. pul.	896 $\pm$ 182 <sup>‡</sup>	936 $\pm$ 108	1076 $\pm$ 96 <sup>‡</sup>

Nitric oxide release was stimulated by calcium ionophore A23187 (10  $\mu\text{mol/liter}$ ) and monitored in situ on the endothelial surface of isolated vascular strips using a porphyrinic based NO microsensor. Data are given as mean  $\pm$  SEM. \* $P < 0.05$ ; vs Young; <sup>‡</sup> $P < 0.05$ ; vs age-matched aorta.

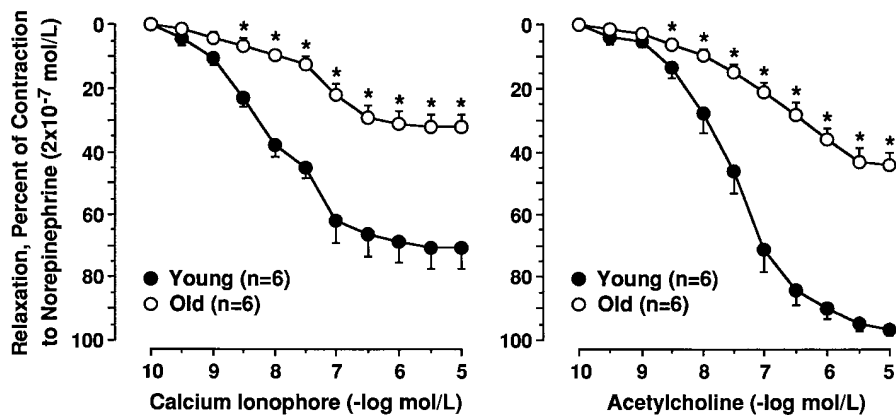


Figure 5. Line graphs showing endothelium-dependent relaxations to the receptor-independent agonist calcium-ionophore A23187 (left panel) and the receptor-dependent agonist acetylcholine (right panel) in aortas of young (5–6 mo old) and old (32–33 mo old) normotensive RORO rats. Vessels were contracted with  $2 \times 10^{-7}$  mol/liter norepinephrine, and then relaxed with  $10^{-10}$ – $10^{-5}$  mol/liter calcium ionophore A23187 or acetylcholine. Relaxations to both agonists were dramatically reduced in aortas obtained from old rats as compared with ones obtained from young rats. Values are means  $\pm$  SEM. \* $P < 0.05$  vs. young rats.

tas obtained from age-matched rats ( $P < 0.0001$ ,  $n = 6$ ). In old rats, however, the initial rate of NO release in pulmonary arteries was significantly faster when compared with the aortas from age-matched rats ( $P < 0.03$ ,  $n = 6$ ).

Fig. 4 (right) presents the average peak NO concentrations in rat pulmonary arteries following stimulation with calcium ionophore A23187 ( $10 \mu\text{mol/liter}$ ). The maximal NO surface concentration increased from  $756 \pm 70$  nmol/liter in young rats to  $940 \pm 124$  nmol/liter in middle-aged rats and  $1032 \pm 145$  nmol/liter in old rats ( $n = 6$  for each age group).

The peak NO concentrations were significantly lower in pulmonary arteries from young rats when compared with the aortas obtained from age-matched rats ( $P < 0.0009$ ,  $n = 6$ ). In old rats, however, the maximal endothelial NO surface concentration was significantly increased in pulmonary arteries when compared with the aortas ( $P < 0.02$ ,  $n = 6$ ).

In pulmonary arteries, the kinetics of NO release as well as peak NO concentrations were unaffected by superoxide dismutase ( $100 \text{ U/ml}$ ; Table II).

## Discussion

In the present study, young (5–6-mo-old), middle-aged (18–19-mo-old), and old (32–33-mo-old) rats have been used in order to delineate the effects of prolonged aging on the kinetics and maximal concentration of nitric oxide (NO) released from the aorta and pulmonary artery using the porphyrinic microsensor recently developed by Malinski and associates (10). After stimulation, the initial rate of NO release and peak NO concentration declined with age only in aortas, as did endothelium-dependent relaxation. In contrast in pulmonary arteries the initial rate as well as peak NO concentration were maintained and even slightly increased with age.

Gains in knowledge of aging in humans have increased demands for suitable animal models, in which the age span is shorter and genetic as well as environmental influences can be better controlled. Although larger mammals have been successfully used in studies of cardiovascular aging, rats are by far the most common model (21), and for good reasons: background knowledge is particularly extensive, their life span is comfortably short (up to 2.5–3 years) and environment and nutrition can be easily controlled. In addition, rats hardly ever become atherosclerotic (this disorder often complicates analysis of human cardiovascular aging) (22). In the present study, rats with an age up to 33 months have been used. Following

the definition by Burek and Hollander, these rats can be taken as representatives of really old animals (21, 22).

NO is a widespread mediator of cell communication involved in different physiological processes (8) and has been implicated in hypertension, diabetes, ischaemia, and atherosclerosis. NO produced by vascular endothelial cells is synthesized by a  $\text{Ca}^{2+}$ -dependent constitutive NO synthase (eNOS) (23). The cell membrane does not present a barrier to the diffusion of NO and is not the rate-determining factor in its propagation between cells. On the membrane of endothelial cells, the concentration of NO is in the range of  $2 \times 10^{-7}$  to  $2 \times 10^{-6}$  mol/liter, and is three to four times higher than that in the cytoplasm (9). From an analytic point of view, the detection of NO in the location with the highest concentration, the surface of the cell membrane, will be the most efficient and accurate method of measuring endogenous NO levels. The short half-life of NO and its loss due to reaction with transition metals or free radicals makes accurate quantitative measurements of NO difficult. Most current methods for NO detection are indirect, relying on measurements of secondary species such as nitrite removed from the biological system, or bioassays that rely on secondary effects (24). The sensitivity and size of the porphyrinic sensor are two important features that permit direct in situ monitoring of NO levels on the surface of cell membranes (9). In addition, the fast response time of this sensor (at least 10 ms) makes it particularly suitable for studying the kinetics of NO release from cells (13, 25).

All forms of cardiovascular disease increase in frequency with age even in the absence of cardiovascular risk factors (5). This suggests that aging per se alters vascular function. In some but not other studies, endothelium-dependent relaxations to acetylcholine decrease with aging (16–19, 26, 27). These studies, however, are difficult to interpret because: (a) several endothelial factors can cause relaxation (20); (b) receptor-signal transduction (i.e.,  $G_i$  protein dysfunction) (28) rather than NO synthase activity may reduce the response; and (c) the response of vascular smooth muscle cells and other target cells may also change with age (16). In the present study, the receptor-independent agonist calcium ionophore A23187 has been used in order to focus on NO synthase activity and not on age-related alterations of receptor-operated signal transduction. The porphyrinic sensor has been chosen in order to directly measure NO concentrations as well as the velocity of NO release. This allowed us to demonstrate that not only peak NO concentration but also the velocity of its release decrease

with age. Since NO has a very short half-life, the kinetics of endothelial NO release are an important determinant of its biological activity. Hence, in addition to a smaller amount of NO produced, a slower release of NO with aging will further reduce the biological effects of this mediator in vascular smooth muscle, platelets and other target cells.

The relatively short half-life (6–50 s) of NO in biological systems might be due to reaction with oxygen, although this decomposition is relatively slow. Superoxide has been established as the main oxidant and scavenger of NO (29). Free oxygen radicals might be important factors in biological aging. Higher formation rates of free radicals from senescent animals observed in isolated biological materials (mainly in mitochondria), accumulation of free radical damage and changes of antioxidant capacities appear to prove the correctness of this assumption (30). In this study, however, reduced NO formation and peak NO concentrations following calcium ionophore A23187 administration in middle-aged and old rats were not normalized by the superoxide scavenger SOD. Thus, superoxide does not contribute to the altered NO release in aortas obtained from senescent rats. As the response to calcium ionophore A23187 and acetylcholine were superimposable in the different age groups, the altered responses of blood vessels to the muscarinic agonist with aging (16) are not related to a dysfunction of the signal transduction pathway, but must involve reduced NO production.

Another important determinant of reduced endothelial NO release in aortas of senescent rats might be altered production of sex hormones. Indeed, endothelium-dependent relaxations are more pronounced in female as compared with male rabbits and gonadectomy diminishes the difference (31). Furthermore, estradiol-17 $\beta$  treatment enhances eNOS mRNA and NOS activity in vascular and nonvascular tissues from female and male rats (32). In the present study, however, plasma estradiol-17 $\beta$  exhibited only minor changes with increasing age. Hence, the age-related differences concerning endothelial NO release in rat aortas are not due to altered hormonal levels.

This study further demonstrates that age differently alters endothelial NO release in rat aortas and pulmonary arteries. Indeed, while NO release decreased with age in the aorta, this response slightly increased in the pulmonary artery. As diastolic, systolic and mean blood pressure did not change with aging, this must be related to chronic exposure to the higher level and/or higher pulse pressure as well as mean flow velocity in the aorta than pulmonary artery (33). Hence, it appears that chronic exposure per se rather than changes of these parameters with age are important. These parameters contribute to structural as well as functional adaptation of the cardiovascular system and in hypertension lead to an increased media to lumen ratio and derangements of endothelial cell function (34). Pulse pressure only increased in the very old animals but NO release already was impaired in middle-aged rats. Although stable with aging, the NO release was lower in the pulmonary artery than the aorta of young rats. PO<sub>2</sub> which is lower in the pulmonary artery than aorta might be involved. Indeed, hypoxia reduces endothelial NO release in rat aortic rings (35) and fetal pulmonary endothelial cells (36) and causes a reduction of cNOS gene transcription in bovine pulmonary artery endothelial cells (37).

Since NO can potentially inhibit several components of the atherogenic process such as vascular smooth muscle cell contraction (1, 2), migration and proliferation (38), platelet aggrega-

tion (39), monocyte adhesion (40) and oxidative modification of low density lipoproteins (41), alterations of the endothelial NO release with age (42) may be of great importance in the atherosclerotic process. Indeed, although intimal proliferation can occur in pulmonary arteries the changes are much less severe than those in the aorta (15).

In conclusion, age differently alters endothelial NO release in rat aortas and pulmonary arteries. After stimulation, the initial rate of NO release and the peak NO concentration significantly declined with age only in aortas, while it increased somewhat in pulmonary arteries. This may be particularly important as NO plays an important protective role by preventing vasoconstriction, alterations in vascular compliance, thrombosis and atherosclerosis.

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