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Anatomically distinct activation of endothelin-3 and the L-arginine/nitric oxide pathway in the kidney with advanced aging

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Abstract

Aging is associated with spontaneous degenerative changes of renal function and structure. The aim of this study was to determine changes of the endothelin (ET) system and NO tissue bioactivity during the physiological aging process. Renal protein expression of ET-1 and ET-3, ET_A, and ET_B receptor mRNA expression, ET receptor binding and distribution, and tissue NO metabolite content were determined in adult, middle-aged, and senescent normotensive female Wistar rats. In senescent animals, medullary ET-3 content increased 3.4-fold ($p < 0.05$ vs. adult), whereas aging did not affect ET-3 levels in the cortex. Local NO bioavailability, determined by NO metabolite tissue measurements, decreased in the cortex only. ET receptor binding capacity—predominantly due to ET_B receptor binding—was lower in medulla than in cortex. Aging had no effect on ET-1 binding capacity or ET receptor distribution, whereas with advanced age gene expression of both receptors decreased. In conclusion, aging causes distinctive expressional changes of the renal endothelin system in otherwise healthy rats. The pronounced increase of endothelin-3 in the renal medulla is associated with preservation of local NO metabolite levels, changes not observed in the cortex. These findings could be important for pathologies and possibly therapy associated with renal aging.

Keywords: Autoradiography; Binding; Endothelin-1; Endothelin-3; ET receptor; Aging; Gene expression; mRNA; Nitrate; NO

Renal aging in humans and rodents is associated with a spontaneous and progressive decline of kidney function and structural changes in medulla and cortex [1,2]. Vasoactive substances, including the endothelins (ETs) [3], have been suggested to play a role in these processes. Endothelins are expressed at a basal level in renal tissue [3,4], contribute to vascular and renal homeostasis [3,5], and their production increases under pathological conditions such as hypertension, uremia, or hyperlipidemia (reviewed in [3,6]). ET-1, the predominant isoform, causes vasoconstriction in the renal cortex while vasodilatation occurs in the medulla [7]. ET-1 modulates cell growth and function in the kidney mainly through the activation of ET_A receptors [3,8], stimulates sodium reabsorption [9], and contributes to salt-sensitivity [10–13]. Moreover, inhibition of endothelin provides a potent means to interfere with the development of renal disease [6]. We have previously shown that both gene and protein expression of ET-1 in whole kidney extracts increases with aging in otherwise healthy, normotensive rats and mice [14,15].

ET-3 differs from ET-1 by six amino acids and is expressed at high levels in renal endothelial and mesangial cells [16–18]. ET-3 is a selective agonist for the ET_B receptor in endothelium and kidney [19], and stimulates

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glomerular cyclic GMP release, an effect that is inhibited by ET_B-selective receptor antagonists [18]. The binding profile of ET-3 along the nephron closely resembles that of ET-1 and is highest in the inner medullary collecting duct [20]. In contrast, cortical and outer medullary collecting ducts as well as glomeruli show only moderate binding of ET-3 [20]. Among other actions, ET_B receptor activation stimulates the release of prostacyclin [21] and nitric oxide (NO), a short-lived vasodilator formed by NO synthases (NOS) [18,19]. Recent work by Garvin and colleagues indicates that the ET_B receptor directly stimulates eNOS expression and inhibits chloride flux in the thick ascending limb [22,23]. Interestingly, defects of the ET-3/ET_B signaling pathway [24] as well as dysfunctional renal NOS [10,11] contribute to salt-sensitivity, suggesting a link between the two systems. Indeed, NO antagonizes the vasoconstrictor and mitogenic actions of ET-1, inhibits ET-1 synthesis, and facilitates sodium excretion (reviewed in [8,25]).

Indirect evidence suggests that alterations of the L-arginine/NO pathway occur with aging. These changes include reductions of circulating NO metabolites [26], changes in basal NO release [27], and NOS isoenzyme expression [28], as well as reduced renal NO metabolite excretion [26]. Whether aging affects local expression of endothelins or NO metabolite tissue content in renal cortex or medulla is not known. In the present study, we therefore investigated the effects of intermediate and advanced aging to study expression and distribution of ET proteins, ET receptor binding and distribution, and measured local tissue levels of the NO metabolites.

Methods

Animals and tissue preparation. Female Wistar rats ($n = 6$–7 per group) aged 6 months (adult), 18 months (middle-aged), and 33 months (senescent) were maintained on regular rodent chow and were used for experiments. Systolic blood pressure was measured in conscious animals using the tail-cuff method [11,29]. Animals were anesthetized (thiopental, 50 mg/kg body weight, i.p.) and sacrificed by exsanguination. Kidneys were excised, decapsulated, snap-frozen in liquid nitrogen, and kept at −80 °C until further processing. Experiments were conducted in accordance with the *NIH Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health* (NIH Publication No. 85-23, revised 1996).

Renal endothelin content. Frozen kidneys were rapidly sliced horizontally and separated between outer medulla and inner cortex as described [11]. Tissues were immediately homogenized for 60 s in ice-cold chloroform:methanol 2:1 containing 1 mmol/L *N*-ethylmaleimide and 0.1% trifluoroacetic acid using a polytron. ET-1 and ET-3 proteins were extracted and quantified using radioimmunoassays and HPLC as described [11].

Determination of renal NO metabolites. Ion-pairing chromatography was used for identification of nitrite and nitrate (NO_x), stable end products of NO oxidation [30], in tissue homogenates as an indirect measure of NO bioactivity [31,32]. Tissue from renal cortex and medulla was homogenized in sterile distilled water, frozen and thawed, centrifuged, and deproteinized by ultrafiltration using a 5 kDa cut-off membrane (Waters). NO_x were quantified by reverse-phase HPLC on an ECE250/4.5 Supersil 100 RP column (Machery Nagel) with photodiode array detection at 210, 215, and 220 nm. Measurements were related to standard curves in the 0–100 μM range generated in the same sample matrix. Injection volumes were 40 μL with a flowrate of 1.0 mL/min. NO_x were quantified by peak height and values were expressed as μmol/g protein.

Gene expression analysis. The Perkin–Elmer 7700 Sequence Detection System (Rotkreuz, Switzerland) was used for quantitative polymerase chain reaction assay, which was performed as described [33]. The probe contains a fluorescent reporter dye covalently linked to the 5′ end and a quencher dye linked close to the 3′ end. Closeness of the quencher to the reporter emitter results in suppression of the reporter fluorescence. During PCR cycling, the probe specifically hybridizes to the corresponding template and is then cleaved via the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase. Sequences of the probes and primers for the rat ET_A and ET_B receptor and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes [33] were designed according to the manufacturer's guidelines and are available on request. The protocols for this procedure, as well as the calculation procedures for quantification, are available at http://www.pebio.com. From mRNA isolated from tissue of whole kidneys ET receptor mRNA expression was compared to the expression of the housekeeping gene GAPDH. All experiments were performed in triplicate. Validation experiments demonstrating that amplification efficiencies of target and reference were approximately equal were performed before using this method for quantification. The amount of target was calculated using the $\Delta\Delta C_T$ method where C_T represents the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The amount of mRNA of the genes of interest was normalized to 1 in the controls.

Autoradiography studies. Distribution of renal ET-1 receptors was identified as described with slight modifications [34]. Briefly, 10 μm serial transverse cryosections of whole kidneys ($n = 5$–7 per group) were thaw-mounted onto gelatinized slides. Endogenous peptide levels were reduced by preincubating sections in 50 mmol/L Tris–HCl buffer, pH 7.4, for 15 min at 22 °C. Slide-mounted tissue was then incubated in Tris–HCl buffer (+5 mmol/L $MgCl_2$, 0.2% bovine serum albumin, and 100 kIU/mL aprotinin) containing 200 pmol/L [^{125}I]ET-1 (specific activity 2000 Ci/mmol/L, Amersham International, Buckinghamshire, UK) for 120 min at 22 °C. ET_A binding sites were identified by incubating kidney sections in presence of the ET_B specific antagonist BQ-788 (150 nmol/L), ET_B binding sites were identified using the ET_A antagonist BQ-123 (150 nmol/L). The degree of non-specific binding was established by incubating alternate slides in the presence of 10 μmol/L unlabelled ET-1. After incubation sections were washed twice for 10 min in Tris–HCl buffer (4 °C), dipped in ice-cold glass-distilled water, and dried in a stream of cold air. Binding was localized at the cellular level by dipping incubated kidney sections in molten nuclear emulsion (Hypercoat LM-1, Amersham International) and exposure for up to 8 days at 4 °C in light-proof boxes. Radioactive slices were also analyzed using a Micro-Imager (Biospace Mesures, Paris, France), where radioactivity is measured by a high resolution digital camera with a spatial resolution of 15 μm. The image (detected light intensity) is converted to a color scale spreading from blue (low radioactivity) to red (high radioactivity). Quantification of ET-1 binding capacity in renal cortex and medulla was performed on cryosections and calculated as cpm/mm^2 [34].

Materials. Unless otherwise indicated, substances were from Sigma Chemical (St. Louis, MO). ET-1 and ET-3 were from Calbiochem/Novabiochem AG (Läufelfingen, Switzerland). Rabbit antibodies against synthetic ET-1 and ET-3 were from Peninsula Laboratories, and radiolabeled [^{125}I]ET-1 and [^{125}I]ET-3 were purchased from Amersham (Amersham, UK). Pentobarbital was from Abbott Laboratories (Chicago, IL).

Statistical analysis. Data are given as means ± SEM, *n* equals the number of animals used. For multiple comparisons, results were analyzed by two-way ANOVA, followed by Bonferroni's correction.

For comparison between two values the unpaired Student's t test, the non-parametric Mann–Whitney U test, or the Wilcoxon signed rank test was used when appropriate. A p value <0.05 was considered significant.

Results

Blood pressure and body weight

Animals were normotensive, and no significant differences in systolic blood pressure were observed between groups (Table 1). Body weight slightly decreased with age ($p < 0.05$ senescent vs. middle-aged, Table 1).

Table 1
Body weight and blood pressure

Group	Adult	Middle-aged	Senescent
Body weight (g)	222 ± 5	$316 \pm 5^{*}$	$294 \pm 4^{*,\dagger}$
Systolic blood pressure (mmHg)	98 ± 3	119 ± 10	112 ± 8

* $p < 0.05$ vs. adult.
† $p < 0.05$ vs. middle-aged.

Endothelin protein content

In adult animals, protein levels of ET-3 and ET-1 were not significantly different between renal medulla and cortex. ET-3 levels were increased in the medulla of middle-aged rats ($p < 0.05$ vs. adult) and further increased of senescent rats (from 112 ± 11 to 381 ± 16 pg/g tissue, $p < 0.05$ vs. adult, Fig. 1A). In contrast, no changes were observed in the cortex (n.s.). ET-1 protein content also increased in the medulla of senescent animals and, to a lesser extent, in the cortex (Fig. 1B, $p < 0.05$). Medullary ET-1 levels were not different between adult and middle-aged animals (n.s., Fig. 1B).

NO metabolite tissue levels

In adult animals, NO_x tissue content was higher in renal cortex than in the medulla (274 ± 27 vs. 191 ± 25 μmol/g protein, $p < 0.05$, Fig. 2A). With senescence, renal NO_x content in the cortex decreased by approximately 50% (from 274 ± 27 to 142 ± 24 μmol/g

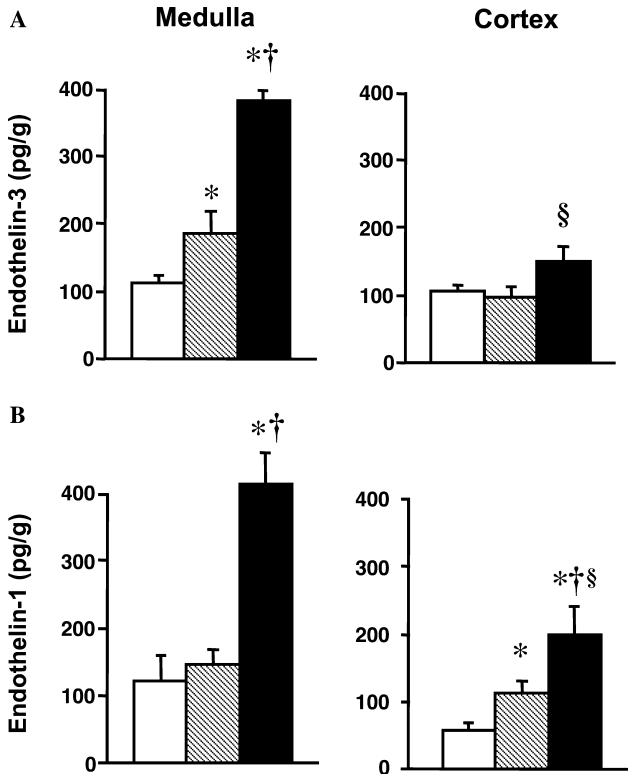

Fig. 1. Tissue protein levels of endothelin-3 and endothelin-1 in renal medulla (left panels) and cortex (right panels). In adult animals, no significant differences in ET-1 or ET-3 expression between cortex and medulla were present. Expression of ET-3 protein increased with age only in the medulla (A). ET-1 protein expression slightly increased in senescent animals in the cortex and, to a greater extent, in the medulla (B). (▨) adult; (□) middle-aged; (■) senescent; $^{*}p < 0.05$ vs. adult, $^{\dagger}p < 0.05$ vs. middle-aged, and $^{\S}p < 0.05$ cortex vs. medulla.

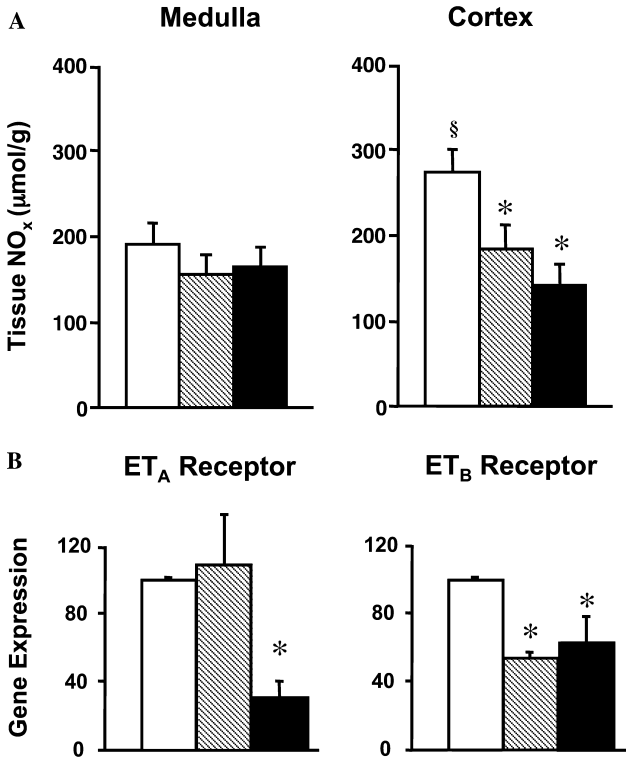

Fig. 2. Tissue levels of NO metabolites in renal medulla (left panels) and cortex (right panels) and renal endothelin receptor mRNA expression. With senescence, NO_x metabolite concentrations remained unchanged in the medulla but were reduced in the cortex (A). Gene expression of both, ET_A and ET_B receptors, was reduced in senescent compared to adult animals (B). Gene expression data are given as percentage of mRNA expression in young animals. (□) adult; (▨) middle-aged; (■) senescent; $^{*}p < 0.05$ vs. adult, $^{\S}p < 0.05$ cortex vs. medulla.

protein, $p < 0.01$ vs. adult), while aging had no effect on NO metabolite levels in the medulla (Fig. 2A).

Endothelin receptor gene expression

Compared with adult animals, expression for both ET_A receptor and ET_B receptor mRNA was reduced in senescent animals to $29 \pm 1\%$ and $63 \pm 15\%$ of adult controls, respectively ($p < 0.05$ vs. adult, Fig. 2B).

Endothelin receptor binding

Binding of [^{125}I]ET-1 was localized to the cortex and tubuli (Figs. 3 and 4). Addition of excess concentrations of cold ET-1 completely prevented binding of the radioactive ligand (data not shown). In the presence of the ET_B antagonist BQ-788, renal binding of [^{125}I]ET-1 was markedly reduced in adult as well as in aged animals ($p < 0.05$, Figs. 3 and 4, middle panels), indicating that with aging ET-1 continues to predominantly bind to ET_B receptors. Aging had only minor effects on receptor distribution. Administration of the ET_A receptor antagonist BQ-123 had only little effect on [^{125}I]ET-1 binding in adult and senescent rats (Figs. 3 and 4, lower panels). ET-1 binding capacity, calculated as cpm per mm^2, was higher in renal cortex compared with medulla ($p < 0.05$) and unaffected by aging (adult vs. senescent; cortex: 878 ± 60 vs. 962 ± 63 cpm/mm^2; medulla: 676 ± 52 vs. 522 ± 98 cpm/mm^2, n.s.).

Discussion

This study demonstrates that in otherwise healthy and normotensive female rodents advanced aging is associated with anatomically distinct changes of intrarenal ET-3 expression and local renal NO metabolite concentrations. Although endothelin receptor gene expression decreased with age, intrarenal distribution and binding capacity of both receptors remained unaffected. To our knowledge, this is the first demonstration of upregulation of ET-3 expression under physiological conditions.

Activation of growth factors, abnormal extracellular matrix synthesis, and hemodynamic alterations have been implicated in age-dependent changes in kidney function and structure [35]. Although activation of the endothelin system has been reported to occur in renal diseases [3,11], little is known about the effect of physiological processes such as aging on expression and intra-

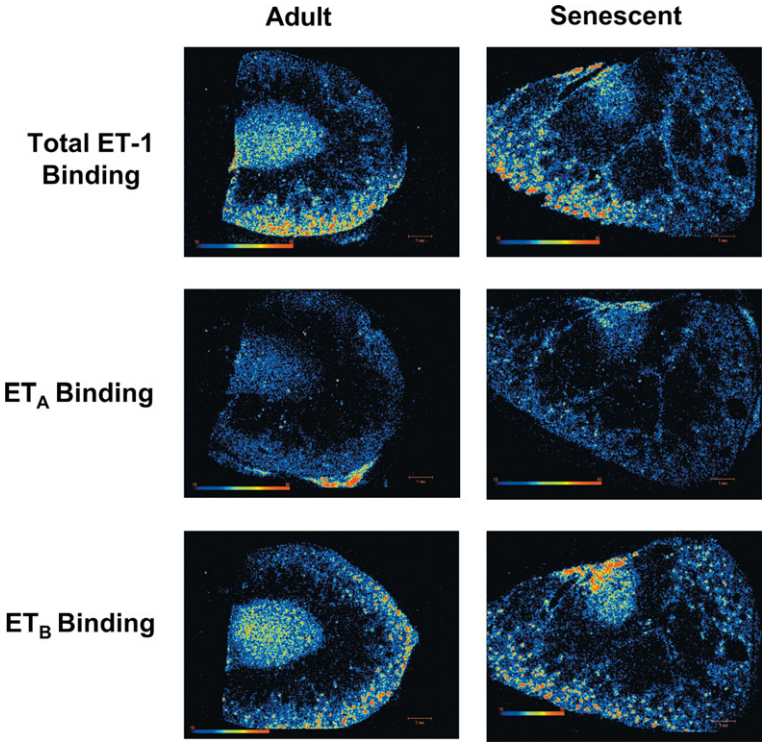

Fig. 3. Aging and renal ET receptor binding. Representative images as detected by Micro-Imager high-resolution digital camera of whole kidney sections radioactively labeled with the non-selective ET receptor agonist [^{125}I]ET-1 alone (top), or in the presence of excess concentrations of ET_B antagonist BQ-788 (middle), or ET_A antagonist BQ-123 (bottom). Left panels: in adult rats, high ET-1 binding is present in the cortex and tubuli (bottom; red "dots" indicate high binding in glomeruli). Addition of the ET_B antagonist BQ-788 markedly reduced ET-1 binding (middle), while the ET_A antagonist BQ-123 (bottom) had little effect on ET-1 binding. Right panels: in senescent rats, distribution of total ET-1 binding and receptor binding was comparable to that of adult rats. Color scale: blue (low radioactivity) to red (high radioactivity). Bar, 4500 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

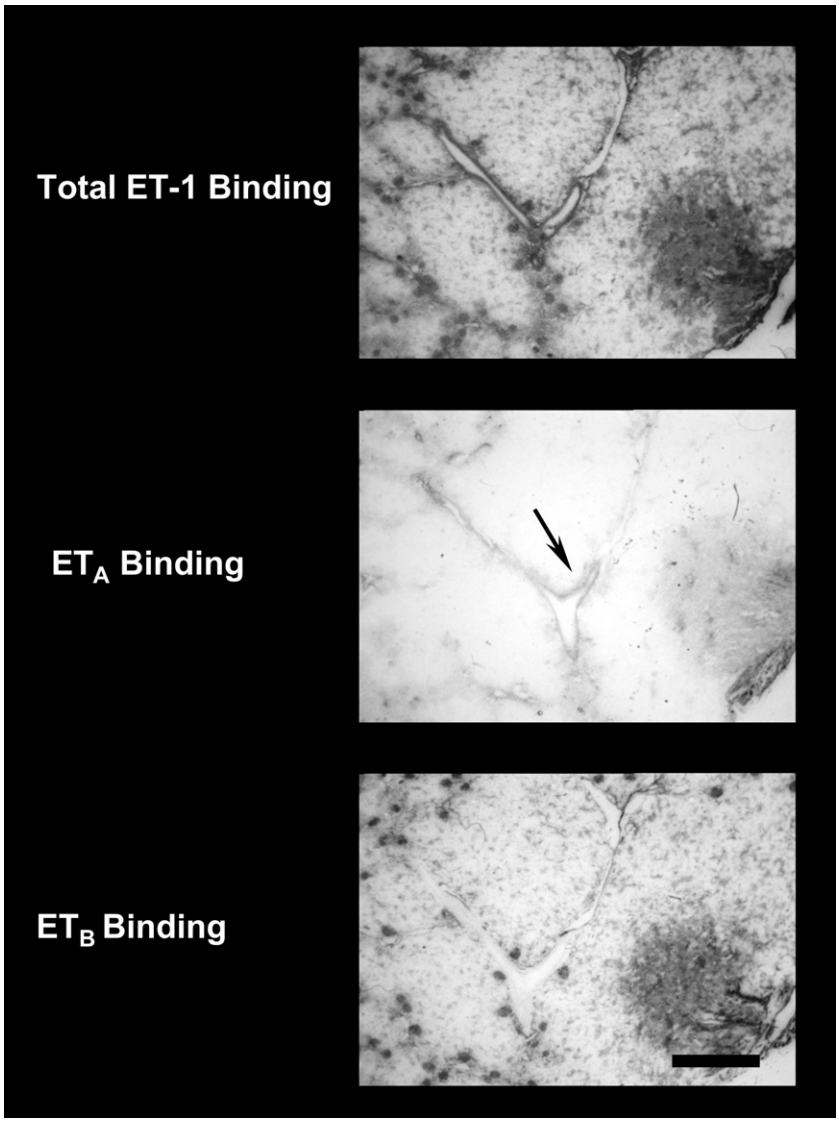

Fig. 4. High-resolution micrographs of ET receptor binding in the kidney of senescent rats. Representative micrographs of renal sections (10 μm) at the border between cortex and medulla using [^{125}I]ET-1 alone (top), in the presence of ET_B antagonist BQ-788 (middle), or ET_A antagonist BQ-123 (bottom), respectively. "Dots" in the cortex indicate high [^{125}I]ET-1 binding to glomeruli. Note discrete ET_A receptor binding of [^{125}I]ET-1 localized to the media of intrarenal arteries (middle, arrow). Bar, 1000 μm.

renal distribution of endothelins and their receptors. In the present study, we found that in adult female Wistar rats ET-3 levels are similar in cortex and medulla. These data are in contrast to a previous study in which we investigated kidneys of male salt-sensitive and salt-resistant Dahl rats [11]. In those experiments, we showed that medullary ET-3 levels were 5–8-fold higher than cortical levels and unaffected by salt-loading [11]. Our present study demonstrates that with senescence—a physiological process devoid of endogenous or exogenous stimuli—ET-3 selectively increases in the renal medulla reaching a level that is comparable to that found in the medulla after salt-loading in rats of the Dahl strain [11].

ET-3 serves as a specific ligand for the ET_B receptor and activates NO synthesis in endothelium and kidney [19]. Since ET-3 has been implicated in regulation of the reabsorption of water through its action on tubular type ET_B receptors [23], it is not unexpected that inhibition or disruption of the ET_B receptor results in hypertension [24,36]. As we have shown here that with advanced aging the ET_B receptor remains the predominant ET receptor in the kidney [37], the observed age-dependent increases of intrarenal ET-3 could provide a physiological buffer mechanism to counteract changes promoting vascular hypertrophy and hypertension seen with aging. These include increased ET-1 expression in the vasculature and kidney [27].

Because of its stimulating effect on NO activity via the ET_B receptor, the selective increase of ET-3 observed in the renal medulla of senescent rats might play a role for the L-arginine/NO pathway. We have previously

shown that in the aorta of aged rats endothelium-derived bioavailability of NO is reduced [27,38]. In contrast to these studies [27], Hill et al. [28] observed enhanced renal vasoconstriction in response to acute NO inhibition in aged rats suggesting enhanced NO release in the renal artery. In the same study, however, a reduction in local NO bioavailability was suggested based on the reduced urinary excretion of stable NO metabolites [26,28]. Collectively, these experiments did not answer the question whether a decrease of NO occurs also locally within the kidney. We have therefore measured intrarenal NO metabolite levels using ion pairing chromatography. The results show for the first time that changes of tissue NO levels occur in the aging kidney in an anatomically distinct pattern. A decrease was observed only in the renal cortex, suggesting that aging selectively and locally impairs intrarenal NO bioavailability. Since NO inhibits transcription and protein synthesis of ET-1 in vitro [39,40], and chronic deficiency of NO enhances ET-1 production in vivo [11], reduced activity of the L-arginine/NO pathway in the cortex may—at least partly—contribute to activation of renal ET with aging. However, it is unlikely that the observed local changes in NO bioactivity are the primary mechanism underlying the age-dependent ET-1 activation, since ET-1 upregulation of similar magnitude was observed in both cortex and medulla when compared to adult animals.

There is no information available regarding the effects of advanced aging on renal endothelin receptor expression and receptor distribution within the kidney. We therefore measured renal ET receptor gene expression, receptor distribution, as well as receptor binding capacity. Using autoradiography we confirmed previous studies that found the ET_B receptor to be the predominant receptor in the kidney of adult rodents [37]. We now extend these observations by showing that distribution and binding capacity of ET_A and ET_B receptors remain unaltered even at an advanced age. In a most recent study, Yanes et al. [41] investigated renal mRNA expression of ET receptors in female spontaneously hypertensive rats at 16 months of age. These investigators observed downregulation of both ET receptors in hypertensive animals, whereas in our study on normotensive rats at a comparable age of 18 months only ET_B gene expression was reduced. With advanced age, however, we also found downregulation of both ET receptors. These findings are in line with reduced functional activity of ET receptors demonstrated by the attenuation of vascular contractility in response to exogenous ET-1 with aging [27]. Reduced receptor activity in aged arteries may be due to the elevated endogenous ET-1 levels [15], a mechanism that is likely to also play a role in the aging kidney. Indeed, down-regulation of ET receptors has been previously demonstrated in the presence of elevated ET-1 levels [42], possibly reflecting a compensatory mechanism to limit local and deleterious increases in peptide expression regulated through ET receptor-mediated autocrine loops [43].

We recently demonstrated that ET-1 is an important determinant of the "degenerative" changes associated with renal aging previously thought to be inexorable [44]. Short-term ET_A receptor blockade partially reversed preexisting glomerulosclerosis and proteinuria involving restoration of podocyte structure [44]. Interestingly, the effects of treatment were confined to glomeruli only and had no effect in the tubulointerstitium [44]. This suggests that aging processes in the kidney in cortex and medulla occur independent of each other. The present study provides additional information supporting this hypothesis, showing that advanced aging is associated with specific expression patterns of intrarenal ET-3 and distinct changes of local NO activity. The relevance of these findings for the different functional and structural pathologies in renal medulla and cortex associated with aging and the possibility of therapeutic intervention under normotensive [44,45] or hypertensive conditions [41] should be further explored in future studies.

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