Sexual dimorphism in glomerular arginine transport affects nitric oxide generation in old male rats

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Schwartz IF, Chernichovski T, Krishtol N, Grupper A, Laron I, Schwartz D. Sexual dimorphism in glomerular arginine transport affects nitric oxide generation in old male rats. Am J Physiol Renal Physiol 297: F80–F84, 2009. First published May 6, 2009; doi:10.1152/ajprenal.00020.2009.—Animal models suggest that decreased renal endothelial nitric oxide synthase (eNOS) activity in old males promotes renal injury, whereas females are protected. We aimed to explore whether aging alters glomerular arginine uptake by CAT-1, the selective arginine supplier to eNOS in rats. Arginine uptake by glomeruli from young males (3 mo) was significantly higher than in young females. Old males (19 mo) exhibited a significant decrease in arginine transport compared with young males, whereas no differences were observed between old and young females. CAT-1 abundance remained unchanged in all experimental groups. The abundance of PKCα (CAT-1 inhibitor) was significantly augmented in young females vs. young males, old vs. young males, and in old females vs. old males. No differences in PKCα content were detected between old and young females. Phosphorylated PKCα was significantly increased in old rats from both genders. α-Tocopherol, a PKC inhibitor, produced a significant increase in arginine transport and restored NO generation in old males only. Ex vivo incubation of glomeruli from old males with PMA (PKC stimulant) significantly attenuated the effect of tocopherol on arginine uptake. In conclusion, attenuated glomerular arginine transport by CAT-1 contributes to the age-dependent, NO-deficient state in old male rats through upregulation of PKCα. In old females glomerular arginine transport is protected from the effects of PKCα by an unknown mechanism.

Glomerular filtration rate; renal vasoconstriction

GLOMERULAR FILTRATION RATE (GFR) will fall with advancing age in most humans and rats (3). The functional decline is secondary to falls in renal plasma flow due to renal vasoconstriction (11). Reduced availability of nitric oxide (NO) has been suggested to play a role in age-related decrease in GFR (4). Several studies demonstrate that this process is characterized by marked sexual dimorphism with females being protected. It is well-known that men with kidney disease progress to end-stage renal disease at a much higher rate than women (9). In healthy men, GFR begins to decline in the forth decade and this is delayed and attenuated in women (12). Animal models support the notion that renal constitutive NO synthase (NOS) activity and protein abundance are reduced in the male kidney with advancing age, as renal injury develops, whereas females are protected (4). Several mechanisms have been proposed to explain the NO-deficient state associated with aging, among which are elevated plasma asymmetric dimethylarginine (ADMA) levels (a competitive NOS inhibitor) as found in the aging male rat (22) and in healthy old males (10), increased oxidative stress, and formation of advanced glycation end products (20). Baylis and co-workers (14) reported that both systemic and renal arginine concentrations were unchanged in old Sprague-Dawley male rats and therefore concluded that substrate availability does not contribute to the aging-related, NO-deficient state. In contrast, we hypothesize that delivery of transported arginine to membrane-bound endothelial (eNOS), selectively by the cationic amino acid transporter-1 (CAT-1) rather than intra- or extracellular arginine concentration, is the predominant factor governing eNOS activity. Indeed, we previously showed in several different animal models characterized by endothelial dysfunction and decreased eNOS activity that CAT-1 activity is attenuated (8, 15, 18, 19). The predominant mechanism resulting in diminished CAT-1 activity in all these experiments was associated with posttranslational modulation of CAT-1 by protein kinase C α (PKCα) (8, 15, 19). The experiments reported herein were designed to test the hypothesis that aging is accompanied by alterations in glomerular arginine uptake by CAT-1, leading to decreased eNOS activity, and to elucidate a molecular mechanism to explain these observations.

METHODS

Materials. All standard reagents were obtained from Sigma, unless indicated otherwise. L-[3H]Arginine was supplied by PerkinElmer (Life and Analytical Sciences, Boston, MA).

Animals and surgical preparation. All animal experiments described in this study were conducted according to the guide of care and use of animals protocol approved by the Institutional Committee on Ethics in animal experiments. Studies were conducted in Wistar rats purchased from Harlan Israel at the age of 3 wk. Animals were then aged at our animal facility and allowed ad libitum access to standard rat chow and tap water until death at ages 8–10 wk (young) and 19 mo (old). Additional animals were given injections of either α-tocopherol, 90 mg/kg body wt ip, every other day, or castor oil as a vehicle, starting 2 wk before death. Creatinine clearance was measured before death, in all experimental groups, as previously described (6).

L-Arginine uptake by freshly harvested glomeruli. Isolation of glomeruli and arginine uptake measurements were performed essentially as previously described (17). In brief, glomerular suspensions from the various experimental groups were incubated and shaken for 10 min in HEPES buffer at pH 7.4, 37°C. L-[3H]Arginine and L-arginine, in a final concentration of 1 mM, were added to a total volume of 1 ml for an additional 1 min. Transport activity was terminated by rapidly washing the cells with ice-cold PBS buffer (4 times, 2 ml/tube). The glomeruli were then dried and solubilized in 1 ml of 0.5% SDS in 0.5 N NaOH. Seven hundred microliters of the
lysate were used to monitor radioactivity and the remaining 300 μl were used for protein content as described previously. Results are expressed as means ± SE of at least five different experiments.

Assessment of cGMP generation. Glomerular cGMP generation was determined by ELISA. Isolated glomeruli were suspended in HEPES buffer supplemented by a phosphodiesterase inhibitor (3-isobutyl-1-methyl-xantine; 1 mM) included to inhibit cGMP degradation. Aliquots were incubated and shaken at 37°C for 10 min after which they were subjected to carbamyl choline (100 μM), a selective eNOS agonist for an additional 5 min. Following incubation, the samples were snap-frozen and then homogenized in 5% trichloroacetate (TCA) at 4°C. The precipitate was removed by centrifugation (3,000 rpm, 10 min) and TCA was ether-extracted. Residual ether was removed by heating the samples for 5 min at 70°C. The samples were then processed for measurement of cGMP by ELISA kit (R&D Systems). Each experiment was repeated four times.

Protein quantification by Western blotting. Glomerular CAT-1, PKCα, and phosphorylated PKCα were determined by immunoblotting. Briefly, freshly harvested glomeruli were separately placed in ice-cold PBS lysis buffer (pH 7.4), containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 4.5 μM leupeptin, and 5 μM aprotinin; ICN Biomedicals), 0.01% Triton X-100, and 0.1% SDS, then mechanically homogenized and left on ice for 45 min. Homogenates were subsequently centrifuged (13,000 rpm for 10 min, at 4°C). Cell lysates were stored in aliquots in −70°C. A membrane fraction was obtained by adding to the pellet an equal volume of lysis buffer supplemented by Tween 20 (0.25%) to solubilize. The protein content of each sample was determined by the method of Lowry. Equal amounts of protein (30 μg) were prepared in sample buffer (2% SDS, 0.01% bromophenol blue, 25% glycerol, 0.0625 M Tris•HCl, pH 6.8, 5% mercaptoethanol) and analyzed on a 7.5% SDS-PAGE gel. The gel was transferred onto Hybond ECL nitrocellulose membranes (Amersham), and blocked in PBS-T containing 5% nonfat dried milk, at room temperature. Membranes were then incubated with polyclonal rabbit anti-rat CAT-1 antibodies, 1:500 (synthesized by Dr. O. Leitner, Weizmann Institute, Rehovot, Israel), mouse anti-rat PKCα and anti-phospho-PKCα (from Santa Cruz Biotechnology) for 1 h at room temperature, washed, and incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:10,000) in PBS-T for 1 h. Membranes were subsequently washed three times, for 5 min each, in PBS-T. Membranes were then stripped and reprobed with monoclonal anti-β-actin antibodies as an internal control. The reactive bands corresponding to the various proteins were detected by enhanced chemiluminescence (Kodak X-OMAT AR film) and quantified by densitometry. Three animals were utilized for each experimental group.

Statistical analysis. Data are presented as means ± SE. One-way ANOVA was conducted for comparison between groups. Post hoc analysis using LSD algorithm was performed to allocate the source of significance.

RESULTS

To validate our experimental model, creatinine clearance and glomerular NO generation were determined. A significant decline in creatinine clearance was measured only in old vs. young males (Fig. 1). We measured glomerular cGMP generation following stimulation with carbamyl choline (CCh), a selective eNOS agonist, as an index of eNOS activity. CCh-stimulated cGMP levels were significantly higher in young males vs. young females and significantly reduced in old males compared with young males, whereas no difference was observed in NO generation by old vs. young females (Fig. 2).

Next, we wished to confirm that changes in glomerular arginine transport velocities in our experimental groups cannot be attributed, merely, to maturation of CAT-1. Indeed, no differences in arginine transport velocities were found at 6 wk vs. 3 mo (Fig. 3A). Therefore, all following experiments were performed utilizing animals age 3 and 19 mo of both genders. At 3 mo, glomerular arginine transport was significantly higher in males compared with females. At 19 mo, the male rats exhibited a significant decrease in arginine transport compared with young males, whereas no differences were observed between old and young females (Fig. 3B).

To determine whether the effects of age and gender on arginine uptake are associated with parallel directional changes in CAT-1, we examined CAT-1 protein levels. CAT-1 protein was identified as ~90 kDa; we found that CAT-1 abundance remained unchanged in all experimental groups (Fig. 4).

To unveil posttranslational effects of aging and gender on CAT-1 activity, hence on arginine transport velocities, we performed Western blotting for PKCα which modulates CAT-1 activity. The abundance of the membrane-bound fraction of PKCα was significantly augmented in young females vs. young males, old vs. young males, and in old females vs. old males, respectively. No differences in PKCα abundance were observed between old and young females (Fig. 5). The relative fraction of phosphorylated PKCα (p-PKCα) was significantly increased in old rats of both genders compared with the respective young ones (Fig. 6). To support the hypothesis that upregulation of PKCα provokes a decline in arginine transport, rats were treated with α-tocopherol, a known inhibitor of PKCα activity (90 mg/kg body wt every other day for 2 wk). Tocopherol administration resulted in a significant increase in arginine transport in old males while such effect was not observed in all other experimental groups (Fig. 7). To establish PKC activation as a possible mechanism for the age-related decrease in arginine transport in old males, we examined the effect of 50 nM PMA, a potent stimulant of PKC, on glomerular arginine transport. Ex vivo incubation of glomeruli with PMA for 30 min significantly attenuated the effect of tocopherol on arginine uptake in old male rats only (Fig. 8). Glomerular cGMP generation in old male rats treated with tocopherol was significantly higher than from similar animals treated by vehicle while no such difference was observed in the other experimental groups (Fig. 9).
DISCUSSION

In this study, the main novel finding is that glomerular arginine transport by CAT-1 declines with age in rat males only. The aforementioned finding leads to attenuated renal NO generation. This may serve as a possible mechanism contributing to the enhanced susceptibility of males to develop age-dependent kidney failure.

The gender-associated differences in arginine transport observed in the current experiments are in accord with observations published by Baylis and collaborators (4). In these two studies, it was found that both CAT-1 and eNOS activity were augmented in young males compared with young females. While in males aging was associated with a significant decrease in both parameters, these remained unaltered in females. The similar directional changes in CAT-1 and eNOS activities, in both genders, provide further support to the hypothesis, raised in our previous publications (8, 15, 17–19), that arginine is selectively introduced to eNOS by CAT-1. Therefore, it is endothelial arginine transport velocity by CAT-1 rather than intra- or extracellular arginine concentrations which governs NO generation by eNOS. How can the data gathered so far explain the notion that males are more “vulnerable” to aging-induced decline in renal function? In young males, which

FIG. 2. Glomerular cGMP generation, from the various experimental groups, following stimulation with carbamyl choline, an endothelial nitric oxide synthase (eNOS) agonist. Results shown are means ± SE of duplicate determinations from 4 separate experiments. *P < 0.05 vs. YM.

FIG. 3. A: uptake of radiolabeled arginine ([3H]-arginine) by freshly harvested glomeruli: a comparison between males and females aged 6 wk and 3 mo. Data are presented as means ± SE of at least 4 different experiments. B: uptake of radiolabeled arginine ([3H]-arginine) by freshly harvested glomeruli from the various experimental groups. Data are presented as means ± SE of at least 5 different experiments. *P < 0.05 vs. YM. #P < 0.05 vs. YF.

FIG. 5. A: representative Western blot analysis showing regulation of PKCα protein level in freshly harvested glomeruli from the same experimental groups. B: densitometric analysis of PKCα contents of the same experiments shown in A. Each bar represents the mean of the relative density units ± SE from 3 different experiments. *P < 0.05 vs. YM. #P < 0.05 vs. OM.
apparently do not exhibit kidney damage, NO generation and arginine transport were in fact higher than in females, whereas no differences were found between old males and females.

While the decrease in arginine transport and NO generation in males can be argued to explain age-related decrease in renal function, the fact that no differences were observed between old males and females is confusing. Baylis and colleagues (4) suggested that with advancing age, the vasodialators present become of increased importance in maintaining renal perfusion in view of the increased renal vasoconstrictor tone due to angiotensin II and renal nerves, implying that NO generation should increase to maintain adequate renal perfusion. Moreover, the renal vasculature of men may be significantly more dependent on NO than that of women (21). Recently, Ahmed et al. (1) showed that with aging the renal vasculature of men becomes more dependent on NO compared with that of women. Taken together, these data suggest that with advancing age males require higher NO generation rates than females to maintain renal vascular tone. Inability to augment glomerular arginine uptake and subsequently NO synthesis results in decreased renal function.

We tried to elucidate a molecular mechanism to explain our findings. The fact that glomerular CAT-1 protein content remained similar in all experimental groups strengthens the hypothesis that CAT-1 activity is primarily regulated at a posttranslational level. A possible involvement of PKC in the regulation of L-arginine transport in different cell types has been discussed for the last several years. In brief, there are two lines of evidence suggesting that PKC participates in the...

Fig. 6. A: representative Western blot analysis showing regulation of phosphorylated PKCα protein level in freshly harvested glomeruli from the same experimental groups. B: relative IOD ratios of phosphorylated PKCα to PKCα in freshly harvested glomeruli from the various experimental groups. Each bar represents means ± SE from 3 different rats. *P < 0.05 vs. corresponding young animals.

Fig. 7. Effect of α-tocopherol (90 mg/kg body wt every other day for 2 wk), a PKCα inhibitor, or castor oil as a vehicle, on arginine transport in freshly harvested glomeruli from different experimental groups. Data are presented as means ± SE of at least 5 different experiments. *P < 0.05 vs. vehicle-treated OM.

Fig. 8. Effects of PMA on glomerular arginine transport in tocopherol-treated rats. Freshly harvested glomeruli from animals, pretreated with tocopherol (a PKC inhibitor; 90 mg/kg body wt ip every other day for 2 wk), were incubated with 0.1% DMSO as a vehicle or PMA, a PKC activator, 50 nM (in 0.1% DMSO) for 30 min. Data are presented as means ± SE of 5 different experiments. *P < 0.05 vs. corresponding vehicle.

Fig. 9. Effect of α-tocopherol (90 mg/kg body wt every other day for 2 wk), a PKCα inhibitor, or castor oil as a vehicle, on glomerular cGMP generation following stimulation with carbamyl choline, an eNOS agonist. Results shown are means ± SE of duplicate determinations from 4 separate experiments. *P < 0.05 vs. vehicle.
regulation of CAT-1 activity. First, according to a model by Albritton and his colleagues (2), CAT-1 protein contains three putative sites for phosphorylation by PKCα, localized in the fifth and sixth intramolecular loops. Second, both CAT-1 and activated PKCα have been reported to be localized in the caveola, allowing for the possibility of CAT-1 and PKC interaction (13).

We recently reported, in three different experimental models characterized by diminished arginine transport, namely hypercholesterolemia, renal failure, and pregnancy, a posttranslational regulation of CAT-1 which was associated with upregulation of aortic PKCα (8, 15, 19). Moreover, during pregnancy, treatment with α-tocopherol, which inhibits PKC, prevented the decrease in arginine transport (15). We decided to focus exclusively at the membrane fraction of PKCα since this is where the interaction between that enzyme and CAT-1 occurs. In glomeruli harvested from old males, the abundance of membrane-bound fraction of both PKCα and its activated form, p-PKCα (7), was significantly increased compared with young animals. Furthermore, when these old male rats were given α-tocopherol to inhibit PKCα activity, the decline in arginine transport was prevented and local NO generation was restored. In addition, ex vivo exposure of glomeruli harvested from tocopherol-treated old male rats to PMA (a PKC stimulant) abolished its beneficial effect. Taken together, these observations, in the aging male rat, are in accord with our earlier reports in renal failure, hypercholesterolemia, and pregnancy. In these models activation of PKCα is a dominant mechanism responsible for the diminished arginine transport and local NO generation. In contrast, in both young and old females, the protein content of the membrane-bound fraction of PKCα was significantly augmented, while p-PKCα was increased in old females only. Moreover, PKCα inhibition by tocopherol and it’s activation by PMA had no effect on arginine uptake velocities in both young and old females. These results imply that although increased translocation of PKC occurs in both young and old females and enhanced activation via phosphorylation is present with advancing age, in females, PKCα does not affect arginine delivery to eNOS through CAT-1. It is conceivable to hypothesize that other processes counteract the inhibitory effects of PKCα on CAT-1, thus preserving glomerular eNOS activity in the aging female. Among other activities, estrogens exert their effect on the vasculature through activation of mitogen-activated protein (MAP) kinase pathways (16). p42/p44 MAP kinase pathway has been shown to directly augment α-glucose-induced arginine transport in human umbilical vein endothelial cells (5). Additional studies are required to reveal whether the aforementioned pathways can be used to explain our observation.

In conclusion, attenuated glomerular arginine transport contributes to the age-dependent, NO-deficient state in male rats. The mechanisms contributing to the aforementioned findings involve activation of PKCα. Glomerular CAT-1 activity in females is reserved by another process yet to be discovered.

REFERENCES


